THE ROLE OF GONADAL STEROID FEEDBACK IN THE REGULATION OF THE ONSET OF PUBERTY

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ABSTRACT

This thesis examines the development of the feedback sensitivity between estrogens and LH in female rats. There are three major objectives: 1) to obtain, using a new radioimmunoassay technique, the pattern of circulating estradiol after birth and through puberty, 2) to test the magnitude of LH response to castration at various ages, and 3) to examine the negative feedback effect of a given blood estrogen level on LH release at various ages.

High levels of "estradiol" were measured in intact rats prior to 30 days of age. The plasma estrogen measured in 5 day-old rats was already elevated and continued to rise to a peak on day 15; circulating "estradiol" then declined, but the 20 day value was still some 10 times the highest value reported for this hormone during the estrous cycle. Plasma estrogen declined to low levels by 30 days and was maintained at these levels through puberty and adulthood. Some circulating "estradiol" persisted in the circulation after bilateral ovariectomy. The level of this non-ovarian substance was greater in young rats with the highest control values for "estradiol". Combined removal of the ovaries and the adrenals, on the other hand, caused the circulating "estradiol" to decline to values below the sensitivity of the assay. Since the administration of a given dose of estradiol benzoate in ovariectomized or ovariectomized and adrenalectomized rats led to similar plasma LH responses, it was concluded that the adrenal's contribution to the "estradiol" levels measured in these experiments was not biologically active as an estrogen.

The postcastration rise in plasma LH offers an index of the sensitivity of the hypothalamic-pituitary system to feedback inhibition by the gonadal steroids. When plasma levels of LH were measured after bilateral ovariectomy, 5 day-old rats did not show any rise 24 hours after castration.

There was a statistically significant rise at all other ages that were studied. The highest rise in plasma LH, with respect to the intact controls, was observed in 15 day-old rats. There was a steady decline in the LH response to castration after day 15, with smallest increment observed in 80 day-old adult rats. These results indicate that there is no negative feedback between estrogen and LH at age 5 days in female rats; 10 day-old rats are the first age group showing the existence of negative feedback, with a peak response on day 15.

The response of the LH release mechanism to estradiol replacement in castrated rats is also a good index of sensitivity of the hypothalamic-pituitary system to estrogens. No significant suppression of LH secretion was observed prior to 10 days of age in prepubertal female rats. Feedback sensitivity was observed to increase after day 10 and reach a maximum by day 15 of age; thereafter there was a decline in feedback sensitivity which continued through puberty.

Since the adrenals were suspected of contributing to the values measured for circulating estradiol in the intact and the ovariectomized rats, comparison of feedback sensitivity was made between a prepuberal and an adult group of rats after combined removal of the ovaries and the adrenals. Comparable blood estrogen level caused a significant decline in plasma LH in the 20 day-old rats, whereas it failed to significantly suppress LH release in the 80 day-old group. Assuming that the binding of estradiol to plasma proteins does not change, as reported in the literature, these data suggest the existence of a difference in sensitivity of immature and adult rats to circulating estrogens.

Taken as whole, this study shows that the young female rat is more sensitive to feedback inhibition of LH release by estrogen than the adult rat. Whether this differential sensitivity to estrogens may account for the onset of puberty cannot be deduced from these experiments. However,

the observation of a gradual decline in feedback sensitivity, starting at 15 days of age and continuing through puberty at 30-35 days of age indicates that the onset of puberty is not due to a sudden decline in feedback sensitivity between estrogens and LH.

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I. INTRODUCTION

Puberty is the stage of development during which reproduction becomes possible. In man, it involves a series of universally recognized stages that occur at approximately the same age within a population (Tanner, 1969; Marshal and Tanner, 1969), whereas in some species, such as the laboratory rat, outward manifestations are lacking.

The study of puberty has been a challenging area of research from the beginning of the modern era of endocrinology. The advent of the technology for the measurement of hormones and the availability of highly purified preparations of pituitary and hypothalamic hormones have provided a deeper understanding of the complex mechanisms involved in the control of puberty. In spite of this, a complete understanding of the physiological changes which occur to cause the onset of puberty has not yet been achieved.

Early reports in the literature established that both the pituitary and the gonads of the immature rat are capable of adult function long before the onset of puberty. This has led to the widely accepted notion that the process of puberty must involve some maturational changes in the central nervous system, especially in areas responsive to gonadal steroid feedback and responsible for secretion of the gonadotropins.

In the sections to follow, an attempt will be made to review the available knowledge on the hormonal changes that occur during sexual maturation; the development of the brain; the responses of the brain to lesions and stimulations; and the feedback effect of gonadal steroids prepuberally. Finally, a series of experiments will be discussed which represent attempts made by the author to contribute to the existing knowledge in the field of sexual maturation.

II. HORMONAL LEVELS DURING DEVELOPMENT:

A comprehensive understanding of the mechanisms controlling the onset of puberty requires a thorough knowledge of how the secretion of pituitary

gonadotropins, the gonadal steroids, and the hypothalamic releasing factors change with age. Any proposed theory explaining the onset of puberty should fully account for the developmental changes occurring in the hypothalamo-pituitary-gonadal axis of the prepuberal animal.

A. Luteinizing hormone (LH) during development:

1. Pituitary LH: The cells of the anterior pituitary of the female rat, at ages as young as 5 to 10 days, seem to possess the capacity to produce gonadotropins (Kragt and Masken, 1972; Siperstein et al., 1954). Dupon and Schwartz (Dupon and Schwartz, 1971) studied the prepuberal patterns of pituitary LH using the ovarian ascorbic acid depletion assay and observed that in the female rat, the pituitary concentration of this hormone reached a peak at around 26 days of age; thereafter pituitary LH declined to low levels at the time of puberty, coincident with vaginal opening and ovulation. Other investigators have obtained similar results in their studies of changes in pituitary LH content during development (Matsuyama et al., 1966; Lisk, 1968;Moore, 1965/66). In the male, the cells of the anterior pituitary seem to develop the capacity for the storage of the gonadotropins some 10 days later than in the female rat (Kragt and Masken 1972; Siperstein et al., 1954). Pituitary LH levels in the male reach a peak at about 60 days of age and then fall to adult levels by 75 days (Lisk, 1968; Dupon and Schwartz, 1971).

2. Plasma LH: The plasma levels of LH in the immature female rat have been studied in detail by several investigators (Fig. 1) and the results obtained are in general agreement (Kragt and Masken, 1972; Ojeda and Ramirez, 1972; Weisz and Ferrin, 1970; Johnson, 1972). Kragt and Masken (Kragt and Masken, 1972) Weisz and Ferin (Weisz and Ferin, 1970) have observed that plasma LH concentration increases from low neonatal levels to a peak at about 15 days of age, thereafter declining to low levels again and remaining so until the time of puberty. Ojeda and Ramirez

(Ojeda and Ramirez, 1972) have shown that the plasma concentration of LH in the female rat is high between 5 and 12 days of age (300-400 ng/ml) with a peak at day 10; the levels then drop to around 125 ng/ml and stay low between day 20 and the onset of puberty with somewhat elevated levels at 25 and 30 days of age.

The results obtained for plasma LH levels in the male rat vary from one laboratory to another. Ojeda and Ramirez (Ojeda and Ramirez, 1972) have observed that the plasma concentration of this hormone rises from already high levels at day 5 and reaches a peak at 12 days of age; the levels drop and remain low from day 15 until day 25 at which time there is a small rise in circulating LH without a peak, which is maintained through day 45 of age. Kragt and Masken (Kragt and Masken, 1972) have obtained a similar pattern but with an apparent lag, so that their early peak is around day 20 and this is followed by a steady decline until day 40 after which a smaller peak was observed on day 50 of life. Weisz and Ferin (Weisz and Ferin, 1970), on the other hand, found surprisingly little change in circulating LH between 5 and 60 days of age; they reported that an occasional animal had a higher level of LH but in the majority, the values were very similar to those in the adult male. Finally, Negro-Vilar et al. (Negro-Vilar et al., 1973) have published data indicating that LH levels underwent a very gradual increase from day 15 to reach a maximum at day 70 which was similar to levels found in the adult male.

3. Hypothalamic luteinizing-hormone releasing factor (LRF): At present, the question of the existence of a common releasing factor for both LH and FSH has not been resolved. The bulk of the data favoring a common RF stems from the use of synthetic material, whereas evidence for separate RFs comes from physiological observations. Due to the existing uncertainty, I will review the literature with the premise that two separate releasing factors exist. Analysis of changes in LRF content of

crude stalk median eminence extracts (SME) from female rats approaching puberty has revealed a gradual rise in LRF activity of the SME beginning day 28 of life followed by a sharper rise just prior to vaginal opening (Ramirez and Sawyer, 1966).

B. Follicle-stimulating hormone (FSH) during development:

1. Pituitary FSH: The concentration of FSH in the anterior pituitary of female rats has been shown to increase from birth and reach a peak at 20 to 25 days of age with a subsequent decrease until the onset of puberty (Corbin and Daniels, 1967; Kragt and Ganong, 1968a; Watanabe and McCann, 1969; Labhsetwar, 1969; Weisz and Ferin, 1970; Suzuki et al., 1971). Watanabe and McCann (Watanabe and McCann, 1969) have observed a further decline in pituitary FSH concentration at the time of vaginal opening without a comparable decline in the content, due to a concurrent rise in the anterior pituitary weight. Various reports on the pituitary FSH levels of male rats are in good agreement with each other (Kragt and Ganong, 1968b; Labhsetwar, 1970; Weisz and Ferin, 1970). The FSH content of the anterior pituitary glands of male rats has been reported (Kragt and Ganong, 1968b) to increase from 17.5 mg (NIH FSH-S-3 equivalents) at 10 days of age to 294 mg at 35 days of age; thereafter it leveled off at about 300 mg and this was observed to persist into adulthood.

2. Plasma FSH: The levels of circulating FSH, already high at 5 days, rise further to a peak at 12 to 15 days of age in female rats; subsequently, plasma FSH decreases (Fig. 1) until the onset of puberty (Ojeda and Ramirez, 1972; Kragt and Dahlgren, 1972). In contrast to LH, the plasma FSH concentration does not show any significant changes at the time of vaginal opening.

FSH has been found to be slightly elevated in the plasma of neonatal male rats (Goldman et al., 1971). A rise in circulating FSH has been reported beginning at day 15 and reaching a peak at 30 and 35 days of

age (Ojeda and Ramirez, 1972; Negro-Vilar et al., 1973; Miyachi et al., 1973; Johnson, 1972); thereafter, the levels fall until a minimum is achieved at day 70, comparable to values observed at day 15 of age (Swerdloff et al., 1971).

3. Hypothalamic FSH-releasing factor (FRF): Measurement of the hypothalamic content of pre- and post-puberal female rats has shown the presence of this releasing factor by at least 10 days of age and at levels higher than any other age studied (Kragt and Dahlgren, 1972). A decline in the hypothalamic FRF activity has been reported by some workers to occur at the time of puberty and vaginal opening (Corbin and Daniels, 1967; Watanabe and McCann, 1969). No report has yet been made on the developmental changes that may occur in the hypothalamic FRF activity of male rats.

C. Prolactin during development:

1. Pituitary prolactin: In contrast to FSH and LH, pre-puberal female rats have a much lower pituitary prolactin content than adult females (Voogt et al., 1970; Minaguchi et al., 1968; Suzuki et al., 1971). The concentration of this hormone in the anterior pituitary is slightly higher in 26 and 31 day old female rats than 21, 33 and 36 day old ones; however, it is not until the first estrus following vaginal opening that pituitary prolactin concentration begins to rise markedly (Voogt et al., 1970).

There are no reports of pituitary prolactin levels in developing male rats.

2. Plasma prolactin: Measurement of serum prolactin in pre-puberal female rats has shown low levels of this hormone during development with a slight rise on the day prior to vaginal opening (Voogt et al., 1970); a subsequent and sharp rise in serum prolactin was observed on the day of vaginal opening without a concomittent increase in pituitary prolactin content. This rise has been attributed to the stimulatory effect of gonadal estrogen secretion.

Plasma prolactin, in developing male rats, has been shown to rise from low levels at 15 and 20 days to a peak at 25 days of age (Negro-Vilar et al., 1973); the 25 day level was maintained until day 50 after which a significant rise occurred up to day 100 of life.

D. Gonadal Steroids during development:

Lack of sensitive methods for the measurement of estrogens in small volumes of plasma has been a deterrent to the attainment of a clear knowledge of the developmental pattern of this hormone in growing rats. Moreover, the existing literature on this subject is far from being in agreement with each other.

In an early study of plasma estrogen levels reported in prepuberal female rats, a fluorometric technique was employed which required pooled blood from 10 to 120 individual animals (Presl et al., 1969). The results of this study show no detectable "estrogens" in the blood of 5 day old female rats; a rise in the level of this steroid at 10 days of age (0.38 mg/100 ml), and no further change up to 55 days of age (0.34 mg/100 ml); the first appearance of circulating "estrogens" was attributed to the 3β-hydroxysteroid dehydrogenase along with follicular cavitation, and the second rise to an increase the level of the above enzyme.

A more recent study employing a radioimmunoassay technique has indicated the presence of extremely high levels of estrogens in developing female rats (Weisz and Gunsalus, 1973); two peaks of "estradiol" were observed, the first at day 10 (mean of 2235 pg/ml) and the second at day 18 of age (mean of 1691 pg/ml). The main drawback to this report is that apparently steroids from the adrenals interferred with the assay, thus making it difficult to interpret the results.

Meijs-Roelofs et al (Meijs-Roelofs et al., 1973) have observed that plasma levels of estradiol-17β are low at 5 days of age but reach a peak at approximately day 15 (60 pg/ml) and then gradually decrease to practically

zero at day 28 and remain so until day 35 which was the last day studied (Fig. 1). However no proof exists for the assumption that these low levels are the true physiological levels of the developing female rats.

Finally, Cheng and Johnson (Cheng and Johnson, 1973/74) have reported a level of 150 pg/ml for estradiol-17β at 10 days of age with subsequent decline in the circulating levels of this hormone down to 18 pg/ml at day 30 of life.

In the male rat, plasma testosterone levels are extremely low during the neonatal period, but rise gradually to adult levels at about the time of puberty (Resko et al., 1968; Knorr et al., 1970).

III. $T_{\frac{1}{2}}$ AND BINDING PROTEINS FOR STEROIDS BEFORE PUBERTY:

In examining the responses of various target tissues to injection of exogenous steroids as well as in the study of the relationship between the endogenous levels of steroids and their responding target organs, one should always consider factors which may have influences upon the effectiveness of that steroid. The biological half-life ($T_{\frac{1}{2}}$) of a steroid as well as its binding properties to various circulating proteins, may influence the extent of the effectiveness of that steroid.

When radioactively-labeled estradiol and testosterone were injected into female and male rats, respectively, similar disappearance curves were obtained for each hormone when injected into adult or immature animals of each sex (Ulrich and Kent, 1968). The implication of this study is that changes in the rates of utilization of these steroids do not contribute to the initiation of puberty.

Brown and Meyer (Brown and Meyer, 1958) have shown that the liver of day-old rat is as capable as that of the adult in causing the in vitro inactivation of three naturally occurring estrogens, whereas the fetal and newborn rat liver is much less effective than that of the adult. On the other hand, Donovan and O'Keefe (Donovan and O'Keefe, 1966) have, by auto-

transplanting ovaries to the spleen and kidney and studying their structure and function, observed that with increasing age the liver appeared to develop the ability to inactivate ovarian hormones; moreover, they suggested that this ability of the liver to metabolize ovarian hormones developed to a significant degree at the time of puberty.

A detailed study of the amount of various steroids bound to the serum of maturing male and female rats has recently been reported (Ramaley, 1971). In the case of the hormones studied, alterations in progesterone binding occurred in both male and female rats at the time of puberty and changes in testosterone binding occurred in females. These changes in the amount of steroid bound to the serum were interpreted to reflect changes in the levels of steroid-binding proteins or the binding properties of these proteins. Of interest in this study is that castration in males and female rats did not alter the pattern of change in steroid binding with age, although it did tend to increase the amount of hormone bound. This increase in the amount of hormone bound after castration may in part be due to the increase in corticosteroid binding globulin (CBG) concentration which is seen in response to the removal of the gonads. Donovan and O'Keefe (Donovan and O'Keefe, 1966) and Donovan et al (Donovan et al., 1967) have suggested that the spectrum of steroids released by the ovaries of the female before puberty is likely to differ, as in the case of testicular steroids, from that produced after sexual maturation. In the growing testes, the capacity for the synthesis and secretion of androgen changes in a qualitative manner as the release of testosterone comes to predominate over that of androstenedione. Work in the rat has shown that at birth the testis avidly converts progesterone to testosterone, but this ability is quickly lost so that by 20 days of age, about 80% of progesterone is converted to androstenediol and only traces of testosterone appear (Steinberger, 1970). Subsequently, the activity of 5α-reductase diminishes and the

formation of testosterone is increased once more. Corresponding changes in the formation of androgen have been described, with the plasma concentration decreasing from birth to reach a baseline level between 10 and 30 days and rising from 40 days onward to adult levels (Resko et al., 1968).

IV. DEVELOPMENT OF THE BRAIN:

Although up to the time of the onset of puberty, the brain undergoes continual maturational changes in both the anatomic and biochemical aspects of its development, certain rather defined stages can be recognized (McIlwain, 1959). All stages of cell division have been observed in the brain prior to birth, but no electrical activity has been detected in the fetal brain. It has been shown in female rats that a significant increase in nuclear volume occurs in the preoptic area and the ventromedial nucleus of the hypothalamus within two days prior to birth (Dorner and Staudt, 1969). This becomes of interest when one remembers that both the preoptic and the ventromedial nuclei of the hypothalamus are involved in the regulation of gonadotropin secretion.

The main growth of neurons and proliferation of axo-dentritic connections occurs during the first 10 days of life. A period of rapid myelination occurs between 10 and 20 days of life; the neural growth and accompanying myelination then continues until puberty (McIlwain, 1959; Jacobson, 1963).

Between 4 and 10 days of age, signs of critical biochemical maturation, as judged by nuclear volume, presence of Nissl substance and dendritic processes, are observed (Jordan et al., 1956; Himwich, 1962). The EEG becomes adult-like at 10 days of age, whereas the attainment of adult levels of most proteins, nucleic acids and lipids occurs before 30 days of age (Himwich, 1962).

As far as the vascular connection between the hypothalamus and the

anterior pituitary is concerned, a functioning hypothalamic-hypophyseal portal system seems to exist in the immature rat. By injection of India ink, it was first demonstrated that portal vessels appeared at 21 to 22 days of fetal life (Glydon, 1957). More recently, it has been shown that the portal blood flow reaches adult levels by the 4th day of neonatal life (Florsheim and Rudko, 1968).

V. BRAIN AND THE ONSET OF PUBERTY:

Damage to various parts of the brain of infants has long been known to cause sexual precocity in human beings (Horowitz and van der Werff ten Bosch, 1962); this effect has been attributed to interference with the feedback action of sex steroids upon the brain (Donovan and van der Werff ten Bosch, 1959). Many areas of the brain, hypothalamic and extrahypothalamic, have been suggested to exert influences upon the processes of sexual maturation; such claims have stemmed mainly from work involving damage to various sites in the brain of the immature rats and observation of subsequent effects on the time of the onset of puberty.

A. Hypothalamus and the onset of puberty:

The hypothalamus has received the most attention in regard to the onset of puberty. Although many years ago it was proposed by Holweg and Junkmann (Hohlweg and Junkmann, 1932) that a neural center existed which was involved in the control of puberty, it was not until much later that their hypothesis received general acceptance. This gradual acceptance was initiated by the ingenious experiments of Harris and Jacobsohn (Harris and Jacobsohn, 1952). These investigators showed that implantation of immature rat anterior pituitaries under the median eminence of hypophysectomized adult females led to a return of vaginal cyclicity and mating. It is interesting that the degree of precocity appearing in response to hypothalamic lesions seems to be independent of the age at which the lesions are placed. It has been shown by Horowitz and Van der Werff ten

Bosch (Horowitz and van der Werff ten Bosch, 1962) that the advancement of the age of vaginal opening in rats lesioned at 3 or 4 days of age is similar to the advancement in rats operated on the 10th, 14th, or 15th day of age. The implication of this observation seems to be that such lesions interfere with some physiological process that normally occurs shortly before puberty and that their effect is due to the presence of the lesions at a particular stage of development (Critchlow and Bar-Sela, 1967). Many workers have provided back-up support for the hypothalamic involvement in sexual maturation and hence different sites in the hypothalamus have been postulated to be of importance in this regard.

1. Anterior hypothalamic area (AHA): The first investigators to clearly show the involvement of the AHA in the process of puberty were Donovan and Van der Werff ten Bosch (Donovan and van der Werff ten Bosch, 1959; Donovan and van der Werff ten Bosch, 1956); they put forth the concept of neural inhibition of gonadotropin secretion in the immature animal by showing that electrolytic lesions placed in the AHA resulted in premature vaginal opening and ovulation in the female rat. The concept that damage to the AHA of prepuberal female rats leads to precocious vaginal opening has since gained ample support (Horowitz and van der Werff ten Bosch, 1962; Elwers and Critchlow, 1960; Schiavi, 1964; Relkin, 1971a; Meijs-Roelofs and Moll, 1972; Bogdanove and Schoen, 1959; Bloch and Ganong, 1971). AHA lesions usually cause the development of some signs of precocious puberty. The most obvious manifestation of precocious puberty is early vaginal opening which along with other signs such as increased uterine weight, and early formation of corpora lutea has been observed in response to AHA lesions (Horowitz and van der Werff ten Bosch, 1962; Donovan and van der Werff ten Bosch, 1959; Elwers and Critchlow, 1960; Schiavi, 1964; Bogdanove and Schoen, 1959). Of curcial importance for deciding whether or not an early vaginal opening is associated with true

precocious puberty is the study of estrous cycles subsequent to the vaginal opening. Most workers have observed that approximately 2/3 of the estrous cycles are normal when early vaginal opening has been achieved by damage to the AHA; the remaining 1/3 seem to have prolonged estrus periods (Donovan and van der Werff ten Bosch, 1959; Meijs-Roelofs and Moll, 1972; Sherwood and Timiras, 1974).

The site of the effective lesion in the prepuberal female rat is usually the ventral region of the AHA and often includes part or all of the suprachiasmatic nuclei, or the optic chiasm (Horowitz and van der Werff ten Bosch, 1962; Donovan and van der Werff ten Bosch, 1959; Elwers and Critchlow, 1960; Schiavi, 1964; Sherwood and Timiras, 1974).

Precocious vaginal opening has also been observed in response to the interruption of the anterior connections to the hypothalamus using a Halasz Knife (Ramaley and Gorski, 1967); however, this transection of the AHA, just posterior to the suprachiasmatic nuclei, was accompanied by constant estrus and hence was classified as "pseudoprecocious puberty" (Ramaley and Gorski, 1967).

Since destruction of the AHA, by placement of lesions, leads to precocious puberty, one might expect stimulation of this region to be associated with delayed sexual maturation. Although Meijs-Roelofs observed that electrical stimulation of the AHA resulted in the advancement of puberty, she was unable to produce precocious puberty if the time at which the operation was performed fell below 27-29 days of life.

2. Posterior hypothalamic area: Lesions of the posterior hypothalamic area have also been associated with precocious puberty in female rats (Schiavi, 1964; Meijs-Roelofs and Moll, 1972; Gellert and Ganong, 1960; Bogdanove and Schoen, 1959; Bloch and Ganong, 1971). Vaginal opening is advanced by posterior hypothalamic lesions which damage the anterior nucleus, the ventromedial and dorsomedial nuclei (Schiavi, 1964) or the

posterior tuberal region anterior to the mammillary bodies (Gellert and Ganong, 1960). These lesions lead to true precocious puberty. According to Gellert and Ganong, (Gellert and Ganong, 1960) lesions restricted to the mammillary region did not effect puberty.

Isolation of the posterior hypothalamic area, ventromedial and arcuate nuclei, from their posterior, dorsal and lateral connections does not affect the time of vaginal opening or first ovulation (Ramaley, 1967). The implication of this observation is that posterior hypothalamic involvement in the onset of puberty, whatever its mechanism, depends on its anterior input.

3. Median Eminence (ME): The involvement of the ME in sexual maturation and puberty onset is to be expected, since this area is the "final common pathway" for many of the reproductive control mechanisms. ME essentially contains the origin of the portal vessels which feed the anterior pituitary and provide the hormonal connection between the brain and the pituitary. It is to be expected that damage to this area of the prepuberal brain would disrupt normal sexual maturation. Lesions of the ME of immature female rats have led to gonadal atrophy (Bogdanove and Schoen, 1959). Surgical transection of all neural input to the ME of 22 day-old female rats resulted in precocious vaginal opening, followed immediately by the onset of persistent vaginal estrus (Ramaley and Gorski, 1967); this observation was attributed to the sudden transection of inhibitory pathways which hold the medial basal hypothalamus in check prior to puberty.

B. Extrahypothalamic involvement with puberty onset:

Among the extrahypothalamic areas of the brain, the limbic system has been the subject of some controversy in regard to its involvement with the process of sexual maturation. The amygdaloid nuclear complex of the limbic system has received the most attention in studies involving the limbic system and the onset of puberty; however, the results of these

experiments have been far from uniform. Bilateral lesions of the amygdala in immature female rats have been claimed to cause precocious puberty (Elwers and Critchlow, 1960). Interruption of one major pathway of the amygdala to the hypothalamus, the stria terminalis, has been observed to be associated with sexual precocity (Elwers and Critchlow, 1969). Riss et al (Riss et al., 1963) have shown that precocious running activity, which is associated with the onset of puberty follows the bilateral aspiration of the piriform cortex and lateral aspects of the amygdala in one week old rats. In agreement with their lesion work, Bar-Sela and Critchlow (Bar-Sela and Critchlow, 1966) have shown that stimulation of the amygdala delays puberty. Although these studies suggest that the amygdala can influence the onset of puberty, they do not establish whether or not true precocious puberty can result from lesions in this nuclear complex.

In contrast to the previous studies mentioned, two other groups have failed to observe precocious puberty in amygdaloid-lesioned female rats (Relkin, 1971a; Relkin, 1971b; Bloch and Ganong, 1971). Relkin (Relkin, 1971a; Relkin, 1971b) who placed lesions in the amygdala of four day-old rats observed a delay in the onset of vaginal opening whereas Bloch and Ganong (Bloch and Ganong, 1971) who lesioned 21 to 22 day-old rats failed to induce precocious puberty by amygdaloid lesions. However, a definite conclusion based on Relkin's work (Relkin, 1971a; Relkin, 1971b) cannot be reached, since the age at which he damaged the amygdala was much earlier than that of Elwers and Critchlow (Elwers and Critchlow, 1960) or Bloch and Ganong (Bloch and Ganong, 1971). On the other hand, data gathered by Bloch and Ganong (Bloch and Ganong, 1971) is in direct contrast to that of Elwers and Critchlow (Elwers and Critchlow, 1960), who used rats of similar age.

Brain destruction in the male rat has proved ineffective in causing precocious sexual maturation (Bogdanove and Schoen, 1959; Critchlow and Bar-Sela, 1967). It is interesting in this regard that administration of exogenous gonadotropins has also failed to cause sexual precocity in male

rats (Price and Oritz, 1944; Woods and Simpson, 1961). As far as the male rat is concerned, it seems unlikely that any experimental procedure could lead to precocious maturation of the spermatozoa because the spermatogenic cycle in the rat has a duration about as long as the period ob puberty.

The notion that specific brain regions may be involved in the control of the onset of puberty has, over the years, strengthened and modified the idea of Holweg and Junkman (Donovan, 1972) that a "sexual center" exists in the brain and that the negative feedback effect of gonadal steroids upon this area inhibits the secretion of gonadotropins in the immature rat.

VI. FEEDBACK EFFECT OF STEROIDS ON GONADOTROPIN RELEASE DURING DEVELOPMENT:

Numerous studies have suggested that an intact feedback relationship between the ovaries and the pituitary gonadotropin secretion exists before puberty in the female rat. It has been shown that the ovaries of the immature rat secrete sex steroids, and as noted above that the pituitary of immature rat contains (Matsuyama et al., 1966; Kragt and Ganong, 1968a) and can secrete large quantities of LH and FSH (Ojeda and Ramirez, 1972; Kragt and Dahlgren, 1972).

Unilateral castration of immature rats leads to compensatory hypertrophy of the other gonad (Ojeda and Ramirez, 1972), whereas bilateral castration leads to an increase in circulating gonadotropins (Yamamoto et al., 1970; Ramirez and McCann, 1963). A number of studies have been reported which indicate that prior to puberty in the rat, the hypothalamic control of gonadotropin secretion may be more sensitive to feedback inhibition by gonadal steroids when compared with the adult animal (Negro-Vilar et al., 1973; Ramirez and McCann, 1963; Byrnes and Meyer, 1951; Smith and Davidson, 1968; Ramirez and McCann, 1965; Eldridge et al., 1974; Bloch et al., 1974; Steele and Weisz, 1974).

Ramirez and McCann (Ramirez and McCann, 1963) employed the ovarian ascorbic acid depletion assay to measure plasma LH and observed that daily subcutaneous injections of estradiol benzoate in immature and adult ovariectomized rats lowered plasma LH below that found in controls, but immature animals were 2-3 times more sensitive to this effect of estrogen than the adults. The same observation was made when prepuberal and adult male rats were studied (Ramirez and McCann, 1965); 3-4 times less testosterone (per unit body weight) was required to repress the rise of plasma LH in castrated immature rats than in castrated adult rats.

However, after a lapse of several years, the report of a failure to duplicate the findings of Ramirez and McCann (Ramirez and McCann, 1963; Ramirez and McCann, 1965) cast a shadow of doubt on the attractive hypothesis of "feedback sensitivity". Swerdloff et al (Swerdloff et al., 1972) using a radioimmunoassay method for the measurement of plasma LH and FSH observed that upon the injection of estrogen into 5 day-castrated adult and immature rats, no difference in the suppressive ability of the steroid at the two age groups was discernable. This prompted other laboratories to re-examine the widely accepted theory that the immature rat is more sensitive to the negative feedback effect of steroids when compared to the adult.

More recently, other investigators have been able to confirm the earlier observations of Ramirez (Ramirez and McCann, 1963; Ramirez and McCann, 1965) using radioimmunoassay instead of bioassay for the measurement of plasma LH concentration. Steele and Weisz (Steele and Weisz, 1974) infused varying amounts of estradiol into ovariectomized pre- and postpuberal rats and observed that much smaller amounts of estrogen per body unit weight per day could lower plasma LH in the spayed immature rats than was necessary to suppress circulating LH in the adult animal. The same conclusion was drawn by Eldridge et al (Eldridge et al., 1974) from

their study which employed subcutaneous injections of estradiol in oil into spayed young and old female rats; they observed that the immature rat was considerably more sensitive to the estradiol than was the mature rat, in their suppression of both LH and FSH.

Bloch et al (Bloch et al., 1974) tested the response of the immature male rat, relative to the adult, to steroid replacement. They observed that in the immature male rat castrated at 10 days of age, 6-12 mg testosterone propionate (per 100 gm body weight) per day reduced LH levels to normal while in the adult rats castrated at 70 days of age, 25-50 mg testosterone propionate (per 100 gm) per day was required to produce similar inhibition.

These studies support the hypothesis originally set forth by Donovan and Van der Werff ten Bosch (Donovan and van der Werff ten Bosch, 1959) stating that at the time of puberty a reduction in the sensitivity for gonadal steroid feedback inhibition of gonadotropin secretion occurs. As to whether FSH or LH is the gonadotropin involved in the feedback sensitivity hypothesis, no clear answer is yet available; however, since both FSH and LH are required to produce precocious puberty (Gellert et al., 1964), this hypothesis can be taken to suggest that estrogen holds either FSH or LH in check until puberty, but that the other gonadotropin is also present.

As far as the author is concerned, no study has yet been reported in which definite proof of the "sensitivity change" theory has been offered. Although studies in which a constant dose of steroid has been injected in both the immature and adult rats (Negro-Vilar et al., 1973; Ramirez and McCann, 1965) offer strong support for the above hypothesis, they lack measurement of blood levels of the steroid after exogenous administration. There is no evidence that the same dosage of a given steroid, per unit body weight, produces the same blood level of the steroid in rats of

different ages. For various reasons, such as different distributing volume, metabolic clearance rate or plasma binding proteins, the fate of the steroids could differ in the immature and the adult rat. No solid data exist as to the half-life and metabolic clearance rate of biologically active steroids in rats of pre- and postpuberal ages. The only report of a systematic study of sex-steroid binding proteins during development, already mentioned in section III (Ramaley, 1971), has indicated a series of complex changes with no clear relation to puberty in the rat.

VII. LOCALIZATION OF FEEDBACK RECEPTORS-IMPLANTATION DATA:

There is disagreement among different laboratories as to the location of steroid feedback receptors. One of the factors contributing to the wide divergence of results gathered from implantation studies is the need for the use of highly sophisticated methodology. In other words, when one undertakes the task of implanting steroid pellets in the brain, one should be absolutely sure of the extent of spread and the systemic absorption of the steroid. Without this knowledge the interpretation of data obtained becomes impossible. As far as inhibitory feedback in the prepuberal rat is concerned, evidence points to the medial basal hypothalamus. Chronic estradiol benzoate (crystalline) implants in the median eminence area of immature female rats resulted in uterine and ovarian atrophy (Smith and Davidson, 1968); the accompanying vaginal opening was interpreted to suggest systemic release of the steroid. When the implants were acutely placed in the anterior hypothalamic-preoptic area, and not in any other location, they resulted in true precocious puberty; this was interpreted as evidence for positive feedback receptors in the anterior hypothalamic-pre-optic area.

It is of interest that steroid implants in the median eminence region of the medial basal hypothalamus are more effective in prepuberal than puberal rats; this has been demonstrated for testosterone and estradiol

(Smith and Davidson, 1967) and also for progesterone (Smith et al., 1969).

One major criticism against the median eminence implants has been the so-called "implantation paradox" hypothesis. Bogdanove has suggested that estrogen implants in the median eminence region may operate by the release of the steroid in the hypophyseal portal circulation with perfusion of the anterior pituitary (Bogdanove, 1963; Bogdanove, 1964). Intrapituitary implants may be ineffective due to the failure of single implants to perfuse the whole gland. It is of interest in this regard that Palka et al (Palka et al., 1966) have shown that H^3 - labeled estradiol placed in the median eminence region does diffuse to the pituitary gland.

VIII. SUMMARY:

Data have been accumulated on hormonal levels and on the development of various components of the hypothalamic-pituitary-gonadal system during sexual maturation of rats, as well as other species. Most attempts have been directed toward the development of a theory for the induction of the onset of puberty. Unfortunately, no single hypothesis can yet account for all the observations made relative to the onset of sexual maturation. However, many facts have been established. The hypothalamic-pituitary axis becomes essentially functional long prior to the onset of puberty as suggested by reports of the existence of stored gonadotropin-releasing factors in the hypothalamus and ample amounts of gonadotropins in both the anterior pituitary and the plasma of immature rats. The gonads also seem to be able to respond to gonadotropins within 10 days after birth. Finally, the brain-pituitary-gonadal axis also becomes operative at an early age. The problem is to assemble these facts in a logical scheme capable of explaining the normal process of sexual maturation as well as the mechanisms responsible for delayed and precocious puberty.

I. PURPOSE OF INVESTIGATION

The purpose of the research described in this thesis has been: a) to

measure, in detail, the blood estrogen level at various ages, using a new radioimmunoassay technique; b) to test the magnitude of LH response to castration at various ages, both pre- and postpuberally; c) to examine the negative feedback effect of a given blood estrogen level on LH release at various ages; d) to identify the possible source and activity of the "estrogen" producing the high levels measured in young female rats.

The examination of the LH response to steroid removal and replacement at various ages permits the analysis of the possibility of a change in the feedback sensitivity of estrogen on LH secretion during development in female rats. The important aspect in which this study differs from previous reports is that blood level of the estrogen causing feedback inhibition has been determined; it is with such knowledge that one can be more certain whether or not the sensitivity of the immature brain to estrogen is different from that of the adult.

II. MATERIALS AND METHODS:

A. Animals: Female Sprague Dawley rats were obtained from Simonsen Laboratories (Gilroy, California) and maintained on a 14h light-10 h dark schedule (lights on at 05:00, off at 19:00). Rat pellets, obtained from Feedstuffs Processing, and tap water were provided ad libitum; in case of rats which were adrenalectomized, the tap water was replaced with normal saline solution.

B. Surgery: Ovariectomies were performed by making small incisions on each side of the animal. When combined ovariectomy-adrenalectomy was desired, a single incision was made in the skin and the ovaries and the adrenals were removed from small slits made on each side of the spinal column.

Surgery was performed under ether anesthesia, between 10:00 and 12:00 hours; the rats were allowed to recover from the effect of the ether before they were returned to their cages, where they were left for 24 hours. When

adult rats were used, ovariectomy was performed on the morning of the second diestrus day, since it has been reported that gonadectomy at this time leads to the largest early LH rise at 24 hours post-castration, when compared to any other day of the cycle (Tapper et al., 1972).

C. Injections and blood collection: 24 hours after surgery, the rats were weighed and then injected subcutaneously with ether oil or estradiol benzoate in oil. Oil injected rats were kept separated from the steroid-treated rats to prevent any possible cross-contamination. Groups of injected rats were then sacrificed at intervals of 1, 2, 3, or 4 hours after the onset of injection. Rats were lightly anesthetized with ether and blood was withdrawn from the abdominal aorta, using heparinized syringes. Blood was centrifuged and plasma stored at -20° until assayed for estradiol and LH. After exsanguination, the rats were checked for completeness of surgery and those with visible remains of adrenal or ovarian tissues were excluded from the experiment. In experiment 1, the rats were sacrificed by decapitation and trunk blood was collected in 12 ml heparinized centrifuge tubes.

D. Steroid: Estradiol benzoate used in these experiments was obtained from Schering Corporation (Progynon(R) benzoate), Bloomfield, New Jersey. The proper dilution was obtained by addition of the stock solution to sesame oil so that 0.5, 1.0, or 2.0 mg estradiol benzoate was contained in each 0.1 ml of oil; these were stored indefinitely at room temperature.

E. Radioimmunoassays: All hormones were measured by radioimmunoassay techniques. In experiment 1, LH and FSH were measured by using the kits provided by NIH and the values are expressed in terms of the RP-1 reference preparations. The prolactin assay employed in experiment 1 and the LH assay employed in experiments 2 to 5 are briefly described in sections E-2 and E-3. The estrogen assay which was developed in this laboratory is fully described in section E-1.

1. Radioimmunoassay of Estradiol

a. Materials and Reagents: Estradiol Standard: 17β-Estradiol was obtained in powder form from the Sigma Chemical Company. The steroid was dissolved in 100% ethanol to give a final concentration of 100 µg/ml (stock solution A); this was then stored at -20°C for 6-12 months. An aliquot of solution A was dissolved in assay buffer producing a 2 µg/ml solution (stock solution B); this was kept at 4°C for 1-3 months.

From the working solution, appropriate dilutions were made to obtain a set of standard estradiol solutions containing the following amounts of steroid in a 0.1 ml aliquot:

5, 10, 15, 20, 30, 40, 50, 75, 100, 150, 200, 300 and 400 pg.

The above set of 13 standards were stored at 4°C, for 30 days.

Labeled estradiol: Estradiol-17β-6,7-H^3, with a specific activity of 48 curies/mM was obtained from the New England Nuclear Corporation; this was used without further purification.

After receipt, the radioactive steroid was diluted with benzene; ethanol (9:1) obtaining a stock solution of approximately 25 µg/ml; this was stored at 4°C for up to 6 months.

From the stock solution, proper dilutions were made by drying down an aliquot and redissolving it in assay buffer to obtain two working solutions; one working solution containing approximately 10-12000 cpm and another with 800-1000 cpm activity in 0.1 ml aliquots were obtained and stored at 4°C for use in the assay.

Antibody to estradiol: The antiserum used was generously supplied by Drs. V. L. Estergreen and T. M. Nett, Washington State University. This antiserum has been made in sheep to 17β-estradiol-17-hydrogen succinate-bovine serum albumin (obtained from NIH). The antiserum was diluted 1:100 with the assay buffer and stored in 0.05 ml aliquots at -20°C. A final concentration of 1:900,000 resulted in a 50% binding of 10-12000 cpm

of the labeled estradiol.

Sephadex LH-20: Sephadex was obtained in powder form from Pharmacia Fine Chemicals, Inc. and was kept at room temperature. Glass Biomed Mini-columns (New England Nuclear Corporation) with an internal diameter of approximately 10 mm were filled with gel to a height of 9 cm; the solvent used for the preparation of Sephadex gel was benzene: methanol (85:15) and the prepared columns were stored in a jar filled with this solvent and kept at $4^{o}C$.

Charcoal-Dextran Suspension: The suspension was made by dissolving 0.625 grams "Norit A" activated charcoal powder (Matheson, Coleman and Bell) and 0.0625 grams grade D Dextran (Mann Research Labs.) in 100 ml assay buffer; this was kept at $4^{o}C$ for one week.

Assay buffer:

Solution 'A': 27.6 grams NaH_2PO_4,H_2O in 1000 ml distilled deionized water

Solution 'B': 53.61 grams $Na_2HPO_4,7H_2O$ in 1000 ml distilled deionized water

The following ingredients were mixed to produce a liter of assay buffer:

195 ml solution 'A'

305 ml solution 'B'

1 gram sodium azide

9 grams sodium chloride

1 gram gelatin (U.S.P.)

500 ml distilled-deionized water

The pH of the buffer was then checked and brought to the 6.9-7.1 range with 2.5 N NaOH, if necessary. The buffer was kept at $4^{o}C$ until signs of bacterial growth were observed.

Scintillation cocktail: To 1893 ml reagent grade toluene (Mallinckrodt)

was added 375 ml p-Dioxane (Matheson, Coleman and Bell) and 80 ml Liquifluor (New England Nuclear); this was kept at room temperature in a brown reagent bottle.

Solvents: Benzene and methanol were of spectroquality (R) grade (Matheson, Coleman and Bell). Ethanol was 200 proof Gold Shield Alcohol (Rossville). Ether for extraction (Ethyl Ether Anhydrous) was obtained in sealed one-pound cans (Mallinckrodt) and stored at -20°C; fresh cans were opened on the day of extraction.

b. Procedures:

Extraction: To a 50 ml extraction tube was added 0.1 ml of H^3-labeled estradiol (800-1000 cpm) in order that the recovery of the steroid throughout the procedure could be monitered; this was followed by the addition of the unknown plasma (volume depending on the expected steroid content) and mechanical mixing on the magnetic mixer. 100 ml ether from freshly opened cans were then added to each tube and the mixture was agitated on the mixer for 60-90 seconds. The tubes were left in the rack for a mimimum of 10 minutes to allow complete segregation of the organic and aquous phases. These two phases were then separated by freezing the lower aquous phase in dry ice-acetone bath.

The organic phase, containing the steroids, was subsequently evaporated under a stream of dry air in 15 ml conical centrifuge tubes. The dried steroid extract was concentrated at the bottom of the tube by twice washing down the walls of the tube with small volumes of ether.

The final dried residue was redissolved in 0.2 ml benzene: methanol solvent (85:15) and transferred to the Sephadex LH-20 column. An extra 0.1 ml solvent was used to rinse the tube and complete the transfer of the steroids to the column.

Chromatography: The steroid extract was placed on top of the Sephadex LH-20 column and eluted through the gel. Following the steroid load, 6 ml

of solvent was run trhough and eluate containing various steroids other than estradiol was discarded. 4 ml of solvent was then used to elute the 17β-estradiol from the column which was collected into 12X75 glass culture tubes; this estradiol-rich fraction was dried down under air and redissolved in 1.0 ml assay buffer. From this 1.0 ml a 0.2 ml aliquot was removed and placed in counting vials for determination of % recovery and the remaining 0.8 ml was subjected to radioimmunoassay.

Radioimmunoassay: 12X75 mm glass culture tubes were used for the assay. 13 standard estradiol quantities were employed in triplicate to allow the construction of a reliable standard curve. 0.7 ml assay buffer was added to each standard tube to produce a total volume of 0.8 ml equal to that of the unknown fractions. 0.1 ml diluted antiserum was added to each tube, except the "total count" and the "nonspecific binding" tubes. The tubes were agitated before 0.1 ml of labeled estradiol (10-12000 cpm/0.1 ml) was added to them; after addition of the isotope, which brought the total volume to 1.0 ml, the tubes were briefly mixed on the magnetic mixer and then placed at 4^{oC} until the following day.

It was observed that 3-4 hours of incubation was enough for the reaction to reach its equilibrium; however, for purposes of time availability, it was desirable to allow an overnight incubation before the assay was terminated. To separate bound and free labeled steroid, 0.2 ml charcoal suspension was added to each tube and the tubes were agitated briefly, and then left in an ice bath for 30 minutes. The tubes were then centrifuged at 2000 rpm (MSE-Mistral 4L refrigerated centrifuge) for 15 minutes. The supernatant was decanted into glass counting vials (Wheaton) and then mixed with 10 ml of the counting cocktail. The vials were placed in a refrigerated liquid scintillation spectrometer (Packard Model 3390) and counted for 10 minutes or until a maximum of 20,000 counts accumulated.

Calculations: The standard curve was constructed by plotting the %

binding of the standard estradiol tubes (as compared to the total binding, that is, zero steroid) against the amount of the estradiol standard added at each point (Fig. 1). The % binding of the unknown tubes were then transferred to the standard curve and an amount of steroid per tube was estrapolated. The following formula was then used to calculate the pg/ml steroid content of the tube:

$$\text{pg/ml} = \text{pg/tube} \cdot \frac{1}{\%\ \text{recovery}^*} \cdot \frac{1}{0.8} \cdot \frac{1}{\text{volume}}$$

*this is calculated separately for each unknown:

$$\%\text{recovery} = \frac{\text{cpm recovered} - \text{background cpm}}{\text{total cpm added} - \text{background cpm to each tube}} \cdot 100$$

Reference to the work of the following investigators was made in developing the above estrogen radioimmunoassay: Estergreen, V.L., personal communication; Abraham, G.E., personal communication; Abraham, G.E. et al., 1971; Abraham, G.E., et al., 1972; Cameron, E.H.D. and Jones, D.A., 1972; Wu, C.H., et al., 1973.

Proof of the assay: To test the capability of Sephadex LH-20 of separating estradiol from other gonadal steroids, the elution pattern of 2-4 steroid tracers through 9 or 15 cm Sephadex columns was determined (Fig. 3, 4). It was concluded that Sephadex LH-20 columns are quite capable of producing a good separation of 17-β estradiol from other gonadal steroids; the 9 cm column was chosen for routine use. Table 7 presents data on cross-reaction of various steroids with the estradiol antiserum.

Tables 2 and 3 present data on the recovery of known amounts of estradiol added to a charcoal-treated plasma pool and on the measurement of estradiol in plasma of rats in different physiological conditions. The intra-assay coefficient of variation for a pool of plasma from lactating rats measured repeatedly (n=11) was 14% and the interassay coefficient of variation for the same pool sample (n=6) was 12%.

Fig. 5 presents data on the concentration of estradiol in a plasma pool

as a function of dilution; good linearity is observed. The reasons for the 2.0 ml sample showing lower estradiol concentration than expected is most probably due to the fact that only 10 ml ether was used to extract this volume of plasma (5:1) whereas other volumes of plasma were extracted with a 10:1 ratio of ether.

2. Radioimmunoassay of LH: The ovine-ovine rat LH double antibody radioimmunoassay method of Niswender et al (1968) was employed except that phosphate-buffered saline with 0.1% gelatin was used as diluent. The anti-ovine LH serum was generously provided by Dr. G. D. Niswender and diluted 1:40,000. The highly purified ovine LH for radioiodination (^{125}I) was a gift from Dr. H. Papkoff. The second antibody (ovine antiserum against rabbit gammaglobulin) was kindly provided by Dr. E. F. Ellington and diluted 1:60. Rat LH, used as the standard for the assay and also for the recovery experiments, was provided by NIAMD. LH values are expressed in terms of the NIAMD rat LH-RP-1 reference preparation.

Analysis of multiple replicate samples of rat serum pools measured at high, intermediate, and low ranges of the standard curve revealed coefficients of variation ranging from 8-18% for interassay variability, and 5-15% for intra-assay variability. Detectable levels of LH were not found in charcoal filtered rat serum. Interassay variability, determined by recovering 5, 10, or 20 ng NIAMD-rat LH per assay tube in each of 3 separate radioimmunoassays, gave means $\pm$ standard deviations of 5 ± 0.9, 10 ± 1.2 and 20.9 ± 1.7, respectively.

3. Radioimmunoassay of Prolactin: The double antibody radioimmunoassay method of Niswender et al (Niswender et al., 1969) was employed, except that the hormone for iodination, the reference preparation and the antiprolactin antisera were those obtained courtesy of NIH. Prolactin values are expressed in terms of the NIAMD Rat Prolactin-RP-1 reference preparation. Analysis of rat plasma pools revealed an intra-assay co-

coefficient of variation of 11% (n=11) and an interassay coefficient of variation of 15% (n=6).

III. RESULTS:

Experiment 1: Changes observed in organ weights and hormone levels between 10 and 20 days of age in female rats.

The purpose of this experiment was to study, in detail, the reported observation of a 15-day peak in plasma LH and FSH (Kragt and Masken, 1972).

A. Organ weights: Fig. 6 shows the pattern of weight gain in female rats during development and puberty. It is interesting to note that between 5 and 30 days of age the rate of gain in body weight is relatively constant, but the rate accelerates during the 5 day period immediately preceding the onset of puberty. Table 4 shows the weight of various organs from rats 10 to 20 days of age. The anterior pituitary shows a progressive gain in weight from day 10 to day 20, with the pituitaries of 20 day-old rats weighing two times as much as that of 10 day-old rats. The ovaries showed a very gradual weight gain during the period of this study; 20 day-old rats have ovaries which weigh about three times as much as those of 10 day-old rats.

There was a gradual increase in uterine weight between 10 and 13 days of age; thereafter, very little weight gain was observed through day 20 of life.

The adrenals of rats between 10 and 20 days of age showed a very gradual and steady weight gain, with the adrenals of 20 day-old rats weighing about 2½ times as much as those of 10 day-old rats.

B. Hormone levels:

1. Luteinizing hormone (LH): Table 5 shows the developmental pattern of plasma LH as measured daily in female rats between 10 and 20 days of age. There is a great degree of variation between animals of the same age group as can be seen from the large standard errors. Moreover, it is

interesting to see the lack of a simple concentration pattern due to the frequency of hormone measurement and the great variation among individual rats. Although several bursts of activity are apparent in Table 5, the overall picture seems to indicate that plasma LH is elevated from day 10 to day 16, after which the levels start to decline.

Table 5 shows the anterior pituitary concentration of LH in developing female rats between 10 and 20 days of age. There is a progressive increase in pituitary LH concentration until the age of 16 days, thereafter decreasing until 20 days of age. The 20 day value, however, is still more than two times the 10 day value for pituitary LH concentration.

2. Follicle-stimulating hormone (FSH): Table 5 presents the data obtained for plasma LH in female rats 10 to 20 days of age. Plasma FSH levels are already high at day 10 and they remain elevated, with some fluctuation, until day 16; a progressive decline in plasma FSH is observed after day 16, which is maintained through day 20 of life.

Table 5 shows values obtained for the anterior pituitary concentration of FSH. As with the pituitary LH, the pituitary concentration of FSH also rises from low levels at 10 days and continues to rise until day 20 of life.

3. Prolactin: Table 5 shows the results obtained for plasma prolactin in blood of female rats 10 to 20 days of age. The striking point about the plasma prolactin pattern is the lack of any major changes in circulating prolactin levels during the period of observation; the plasma prolactin concentration at day 20 is the same as day 10 of life.

Table 5 presents data on the anterior pituitary concentration of prolactin between 10 and 20 days of life. The pituitary prolactin concentration seems to be increasing during the period of observation, with the 20 day levels being three times as much as the 10 day levels.

Experiment 2: Time course of plasma LH rise in response to ovariectomy.

The purpose of this experiment was to find the shortest interval after ovariectomy at which time the plasma LH levels were significantly elevated.

Table I shows the values obtained for plasma LH is in rat sacrificed 5 and 24 hours after bilateral ovariectomy. Plasma LH remained at near intact values at 5 hours after removal of the ovaries, whereas a nearly significant increase was observed when the animals were sacrificed 24 hours after the operation. Thus, a 24 hour interval was chosen as the interval between ovariectomy and steroid replacement. It has been reported (Tapper et al., 1972) that bilateral ovariectomy, when performed in the morning of the second day of diestrus, will lead to high LH levels in the plasma after 24 hours. When ovariectomy was performed at other days of the cycle, the 24 hour plasma LH response was not appreciable. Therefore, in my experiments, adult rats were ovariectomized only on the morning of their second day of diestrus.

Experiment 3: Circulating estradiol levels after a single subcutaneous injection of estradiol benzoate.

The purpose of this experiment was to find a dose of exogenous estrogen causing the elevation of circulating estradiol to the levels observed during the estrous cycle.

Table 6 presents the results obtained when two different doses of estradiol benzoate were used in adult rats, 24 hours after bilateral ovariectomy. As can be seen, the 5 µg (per 100 grams, body weight) dose of estradiol benzoate resulted in the largest elevation of plasma estrogen; these levels are up to 40 times the values reported for serum estradiol during the rat estrous cycle (Kalra and Kalra, 1974). Of the other doses used, the 0.5 µg (per 100 grams, body weight) dose of estradiol benzoate produced circulating estrogen levels which were more in the range of serum estradiol concentration in intact female rats. Peak estrogen level was produced by 1 hour and maintained until 2 hours after injection, and

before returning to preinjection values (Table 6).

Experiment 4: Response in plasma LH and estradiol levels to ovariectomy and to estrogen replacement.

The purpose of this experiment was to study the effects of estrogen removal and its replacement on the circulating levels of LH and estradiol.

In interest in this series of experiments was the high levels of estradiol measured in intact rats, prior to 30 days of age; the data on plasma estradiol concentration in intact rats, during prepuberal development and adulthood, is presented in Fig. 7. Ovariectomy failed to cause a complete decline in circulating estrogens; this observation was more pronounced in the young rats which showed the highest control values for "estradiol".

A. 5 day-old females: Fig. 8 shows the plasma levels of estradiol and LH in 5 day-old rats, in response to the removal of the gonads and the administration of 0.5 µg estradiol benzoate per 100 grams of body weight.

It is interesting to note the unusually high levels of estrogen in the plasma of intact (750±261 pg/ml) and ovariectomized (459±52 pg/ml) rats of this age. The most striking aspect of this group of rats is failure of circulating LH to decline in response to the removal and the replacement of ovarian estrogen.

B. 10 day-old females: As can be seen from Fig 9 ovariectomy resulted in a significant increase in circulating LH levels by 24 hours with the release apparently continuing for one hour after the administration of estradiol benzoate. By 2 hours after steroid injection, the plasma LH has declined from its one hour peak; however, the decline is not significant until 4 hours after the onset of the injection. Although a decline in plasma estradiol levels is observed after ovariectomy and an increase observed after steroid injection, none of the changes are statistically significant.

C. 15 day-old females: A significant increase in circulating LH

levels is observed in response to ovariectomy, followed by a significant inhibition of LH release one hour after steroid replacement (Fig. 10). The low level observed at one hour is maintained through 4 hours after the onset of steroid injection.

Although plasma estradiol fell to half its pre-operation value, ovariectomy did not result in a statistically significant decrease in circulating estradiol levels ($p=0.66$). Estradiol benzoate administration did not result in a significant increase in plasma estrogen at any point after the onset of injection.

D. 20 day-old females: Fig. 11 presents the LH and estradiol responses to ovariectomy and estradiol benzoate administration in 3 week-old rats. Ovariectomy resulted in a significant increase in plasma LH levels and a significant decline in plasma estradiol levels, 24 hours after the operation. Although circulating estrogen did not show a significant increase at any point after steroid injection, plasma LH levels exhibited steady decline with a significant decrease observed at 4 hours after the onset of estradiol benzoate administration.

E. 25 day-old females: A significant rise in plasma LH levels is observed following ovariectomy (Fig. 12) with a minor additional increase at one hour after steroid injection. A steady decline is seen after the one hour peak with significantly lower plasma LH values seen at 3 and 4 hours subsequent to the estradiol benzoate administration. Circulating estradiol showed a significant drop 24 hours after removal of the ovaries; however, at no time after steroid injection did the plasma estrogen show an increase over the pre-injection value.

F. 30 day-old females: Plasma LH levels rose significantly in response to ovariectomy (Fig. 13) when measured 24 hours after the operation; this increase was maintained for one hour after the onset of the estrogen administration. Although the plasma LH values at 2 hours were 1/3 that

observed at one hour after injection, this decline was not statistically significant.

Circulating estradiol declined insignificantly 24 hours after the removal of the gonads; however, estradiol benzoate administration resulted in a minor, but significant increase in plasma estradiol one hour after injection; the one hour level was maintained throughout the rest of the observation points.

G. 40 day-old females: Fig. 14 presents data on circulating LH and estradiol in response to ovariectomy and steroid replacement in 40 day-old rats. Although plasma estradiol showed an insignificant drop, 24 hours after castration, plasma LH exhibited a significant increase at this time following the operation. Neither LH nor estradiol showed any significant changes in their circulating levels at any point after the estradiol benzoate injection.

H. 80 day-old females: Both plasma LH and plasma estradiol exhibited minor but significant changes in their levels 24 hours after ovariectomy (Fig. 15). The increase in circulating LH continued for one hour after the estrogen injection. It fell thereafter as the circulating estradiol rose significantly and reached a peak by 2 hours. However, there was considerable variation in the response in individual rats, and the decrease was not statistically significant.

Experiment 5: Response of the LH release mechanism and circulating estradiol to ovariectomy and adrenalectomy and to estrogen replacement.

Since ovariectomy alone did not result in a complete decline in circulating "estradiol", and since it has been observed that the additional removal of the adrenals leads to lower plasma "estradiol" levels, it was decided to compare an adult and an immature age group in their feedback sensitivity, after the combined removal of the adrenals and the ovaries.

A. Fig. 16, 17 show results obtained in 20 day and 80 day-old female

rats. Both age groups received 1.0 µg estradiol benzoate (per 100 grams body weight) 24 hours after combined removal of the ovaries and the adrenals.

The 20 day-old animals (Fig. 16) showed a significant decrease in circulating estradiol as well as a significant increase in plasma LH after the surgery. Treatment with oil did not result in a significant change in either the steroid or the LH concentrations of the plasma, when tested 2 hours after the injection. Estrogen injection increased the circulating estradiol levels significantly, and resulted in a 50% reduction in plasma LH levels by two hours; however, this reduction was not statistically significant.

The 80 day-old animals (Fig. 17), while showing a significant reduction in circulating estradiol, did not show a statistically significant rise in plasma LH in response to the combined surgery. The estradiol benzoate injected into these animals resulted in a statistically insignificant rise in plasma estrogen, while no change in circulating LH was observed.

B. To obtain comparable circulating estradiol levels in 20 and 80 day-old animals, 2 hours after steroid injection, groups of rats from each age level were injected with various doses of estradiol benzoate. It was observed (Figs. 18, 19) that the 0.5 µg/100 grams dose in the immature animals yielded plasma estradiol levels comparable to what 1.0 µg/100 grams dose of the steroid produced in the mature animals.

The removal of the ovaries and the adrenals resulted in a significant decrease in circulating estradiol as well as a significant increase in plasma LH levels in 20 day-old rats. Oil did not result in any significant changes in either the estradiol or the LH levels in circulation, 2 hours after its injection. However, 0.5 µg estradiol benzoate (per 100 grams, body weight) while causing a significant increase in plasma estradiol, resulted in a significant decrease in circulating LH levels.

There was a significant decrease in plasma estradiol along with a

significant increase in plasma LH levels in the 80 day-old rats (Fig. 19), 24 hours after the removal of the adrenals and the ovaries. Injection of oil did not produce any significant changes in either the estradiol or the LH levels of the plasma. 1.0 µg estradiol benzoate (per 100 grams, body weight), although causing a significant increase in circulating estrogen, did not result in a statistically significant decrease in plasma LH levels 2 hours after its injection.

IV. DISCUSSION AND CONCLUSIONS:

The original idea behind Experiment 1 was to study, in detail, the reported observation of a 15 day peak in plasma LH and FSH (Kragt and Masken, 1972). Since no data existed on plasma prolactin levels prior to 20 days of age, the measurement of this hormone was also included in the investigation. Body weight, as well as the weight of the sex accessory glands, were also recorded to allow possible interpretation of changes seen in the circulating gonadotropins and prolactin.

Although Kragt and Masken (Kragt and Masken, 1972) have reported a simple plasma gonadotropin pattern between 10 and 20 days of age, the present study revealed that large day to day variations existed in the levels of these hormones.

There seems to be the tendency for a rise in synthesis and/or the storage of the gonadotropins and prolactin (Table 5) in the anterior pituitary between 10 and 20 days of life. The plasma levels of LH and FSH stay elevated as the concentration of these hormones rises in the anterior pituitary, between day 10 and day 16. Although the pituitary concentration continues to rise, the plasma levels of LH and FSH show a decline after day 16. These data are in general agreement with those of Kragt and Dahlgren (Kragt and Dahlgren, 1972) who reported high circulating levels of FSH at 10 and 15 days of age and low levels at day 21. These data are also in agreement with those of Ojeda and Ramirez (Ojeda and Ramirez, 1972)

who observed high plasma LH levels between day 10 and 15 and lower values at day 20 of life. The present results also confirm the data published by McCann et al (McCann et al., 1974) showing high circulating levels of LH and FSH on day 15 and low levels on day 20 of prepuberal life.

The changes observed in plasma and pituitary prolactin concentrations (Table 5) are much less pronounced than those observed for the gonadotropins. In contrast to the pattern of plasma and pituitary levels of the gonadotropins, circulating prolactin levels stay rather constant between days 10 and 20, whereas there is a general increase in pituitary concentration of this hormone during the period of observation. The plasma prolactin data are in good agreement with those reported by McCann et al (McCann et al., 1974) who observed the same values for this hormone at 15 and 20 days of age.

It may be inferred from the present data that between days 10 and 16 of life in the female rat, rapid release of LH and FSH takes place, concurrently with more rapid synthesis and resulting storage of these hormones in the anterior pituitary; after day 15 and through day 20 there seems to be a decrease in the release of these hormones which may be occurring along with the continuation of the increased synthesis rate. The results imply that the hypothalamic releasing factors for LH and FSH are present at this time and able to stimulate both synthesis and release. LRF and FRF have been measured during this period (12 and 16 days of age), and hypothalamic concentration of FRF has been observed to decrease at around 15 days of age (Kragt and Dahlgren, 1972).

The accessory organs (Table 4), in general, showed graduat increases in their weights during the period of investigation. The anterior pituitary and the uteri of these rats showed a doubling, whereas the ovaries and the adrenals showed a tripling of their weights between 10 and 20 days of age. These results are in close agreement

with data reported by Baker and Kragt (Baker and Kragt, 1969) who measured organ weights at 5 day intervals during this period of perpuberal development.

The pattern of body weight gain in growing female rats is presented in Fig. 6. This is in good agreement with data reported by Baker and Kragt (Baker and Kragt, 1969).

To study feedback sensitivity between LH and estrogens, animals of various ages were castrated and 24 hours later injected with a single subcutaneous dose of estradiol benzoate. Plasma LH was measured in groups of rats sacrificed at intervals of 1 hour up to 4 hours after the steroid injection.

The reason for choosing 24 hours as the time interval between ovariectomy and estrogen administration was threefold: one requirement was to have little or no change in estrogen-sensitive receptors of the brain. For this, it was necessary to allow as little time as possible to elapse after removal of the ovaries before testing of feedback sensitivity. It has been reported, for both male and female rats, that not only feedback sensitivity, but also accessory organ responsiveness is reset to a new level several days after castration (Eldridge et al., 1974; Swerdloff et al., 1972; Ramirez and Sawyer, 1974). The second requirement was the need for an elevated circulating LH at the time of estrogen replacement in order that feedback phenomena might be more readily observed. The third requirement was that circulating estrogen levels be low at the time of estradiol benzoate injection, so that the observed feedback could be attributed to the exogenous estrogen.

The satisfaction of the above requirements seemed inherent in the choice of 24 hours after removal of the ovaries; 5 hours after ovariectomy in adult female rats (Table 1), there was no increase in plasma LH. This is unlike the situation in male rats, in which castration leads

to a very rapid rise in plasma LH (Ramirez and Sawyer, 1974). A possible reason the 24 hour postcastration LH rise fell short of statistical significance in the initial experiment is that no attention was given to the stage of the cycle at which ovariectomy was performed. The magnitude of the early LH response to castration in cycling female rats depends on the stage of the estrous cycle during which the operation is performed (Tapper et al., 1972); ovariectomy on the morning of diestrus-2 results in the greatest LH rise, as compared with ovariectomy on any other day of the cycle. Hence, it was decided that the ovariectomy of adult rats (Exp. 4-G,H and Exp. 5-A,B) be performed only on the morning of diestrus-2, between 10 a.m. and 12 noon.

To test the sensitivity of the LH release mechanism to estrogens, the aim was to maintain a constant blood estrogen level that could be reproduced in ovariectomized rats of various ages; this would have been best achieved with the intravenous injection of a dose followed by the constant infusion of the steroid. However, since animals as young as 5 days of age were employed in this study, intravenous catheterization was impractical.

The subcutaneous injection of estradiol benzoate, then, was tested for its feasability as the route of steroid administration (Table 6). Four doses of estradiol benzoate were examined; 5.0 µg estradiol benzoate per 100 grams of body weight produced a huge circulating estrogen level at one hour with a rapid decline by two hours after the injection. These blood levels were extremely high and outside the range of plasma estradiol during the rat estrous cycle (Kalra and Kalra, 1974). The 0.1 to 0.8 µg doses of estradiol benzoate, on the other hand, resulted in circulating estrogen levels which more closely resembled the steroid levels of the intact rat; moreover, the blood levels of the steroid obtained with these doses resembled in intravenous injection, in that

the same blood level was maintained for two hours after the injection (Table 6). It was then decided to employ the subcutaneous estradiol benzoate injection as the route of steroid administration; 0.5 µg per 100 grams of body weight was chosen as the routine dose of the exogenous estrogen in order that the physiological estradiol levels of the intact rat be more closely reproduced.

Of importance in this study were the pattern and the magnitude of circulating "estradiol" observed in intact rats during prepuberal development and adulthood (Fig. 7). The plasma estrogen levels measured in 5 day-old rats are already elevated and continue to rise to a peak on day 15. Circulating "estradiol" then declines, but the 20 day value is still some 10 times the highest value reported for this hormone during the estrous cycle (Kalra and Kalra, 1974). Plasma estrogen reaches low levels by 30 days and is maintained at this level through puberty and adulthood. The high levels of "estradiol" in intact prepuberal rats in the present study are similar to values reported by Weisz and Gunsalus (Weisz and Gunsalus, 1973) who measured the serum levels of this hormone between 5 and 25 days of age. Although Weisz and Gunsalus observed two "estradiol" peaks, at days 10 and 13, the present study revealed only a 15 day peak in the plasma concentration of this hormone. It may be of interest that Meijs-Roelofs et al (Meijs,Roelofs et al., 1973), although reporting very low levels of estradiol, have also observed a 15 day estradiol peak (60 pg/ml) between 5 and 35 days of age.

The high values obtained for "estradiol" during the course of this study raises the question of whether the compound that was being measured was in fact estradiol. Moreover, it was of interest to learn the source of the "estradiol" and its biological activity.

Fig. 20 shows the plasma LH rise, in response to bilateral ovariectomy, in rats of various ages. The 5 day-old rats did not show any

rise in circulating LH levels after ovariectomy. The decline in their plasma LH is not statistically significant and most probably represents random variation.

The increase in plasma LH was statistically significant in all other age groups that were studied (Fig. 20). The highest rise in plasma LH with respect to the intact controls, was observed in the 15 day-old rats 24 hours after ovariectomy. There was a steady decline in the LH response to castration after day 15, with the lowest increase observed in the 80 day-old adult rats. Since removal of the ovaries and hence the ovarian estrogens releases the hypothalamic-pituitary system from feedback inhibition, the postcastration rise in plasma LH offers a good index of sensitivity of the above system to estrogens. From Fig. 20 it is evident that no negative feedback between estrogen and LH exists at age 5 days in female rats; 10 day-old rats are the first age group showing the existence of negative feedback which then reaches a peak on day 15. The absence of negative feedback at age 5 days confirms data reported by Kragt and Masken (Kragt and Masken, 1972). There seems to be a gradual loss of feedback sensitivity after day 15 which continues through puberty.

Fig. 21 shows the response in plasma LH to a single estradiol benzoate injection, expressed in terms of percent change at various hours after injection relative to the pre-injection values. This response to estradiol replacement is an index of sensitivity of the hypothalamic-pituitary LH system to estrogens.

It is evident from Fig. 21 that the 5 day-old rats lack a functioning hypothalamic-pituitary-gonadal negative feedback system; this is supported by the data presented in Experiment 4a, in which it was observed that 5 day-old rats did not show an elevation in their circulating LH following bilateral ovariectomy. Moreover, these results confirm the data of Baker and Kragt (Baker and Kragt, 1969) who, through indirect measurements,

observed no significant suppression of gonadotropin secretion prior to 10 days of age in prepuberal female rats.

The first indication of the existence of negative feedback is observed in the 10 day-old rats, where a significant decline in plasma LH is measured at 4 hours after steroid injection (Fig. 21d). The data in Fig. 21 (a-d) seem to suggest that feedback sensitivity, between LH and estradiol, increases after day 10 and reaches a maximum by day 15 of age; thereafter, there is a decline in feedback sensitivity which tontinues through puberty. Although the 30-80 day-old rats show some evidence of LH suppression between 2 and 4 hours after the steroid injection, none reach the level of statistical significance (Fig. 21 b-d).

Of importance in these results is the observation that only the 15 and 20 day-old groups exhibited the negative feedback phenomenon, at one hour after estrogen injection; this offers further evidence for the greater sensitivity of these rats to estradiol, as compared to the other age groups.

One of the striking observations made in the course of these experiments was that in none of the age groups studied did bilateral ovariectomy lead to undetectable circulating estrogens; this coupled with the observation of high "estradiol" levels in intact young rats, suggested the possible existence of a substance which interferred with the estradiol measurements. Two recent reports bear on this subject; Weisz and Gunsalus (Wwisz and Gunsalus, 1973) have reported large amounts of "estradiol" in serum of immature rats and they have been able to significantly reduce those levels only by combined removal of the ovaries and the adrenals. Competitive protein binding or double isotope derivative assays revealed much lower estradiol values than their routine radioimmunoassay technique. This suggests the presence of an interferring substance, other than estradiol, coming from the adrenals. On the other hand, Shaikh and Shaikh (Shaikh

et al., 1975) have recently studied the pattern of secretion of estradiol in the ovarian and adrenal veins of intact adult rats and have observed, using a supposedly specific antiserum, much higher levels of this hormone in the adrenal than in the ovarian veins. The pattern of release of this steroid from the two glands was identical throughout the estrous cycle.

Since the adrenals were suspected of contributing to the values measured for circulating estradiol in the intact and ovariectomized rats, it was decided to compare sensitivity to feedback inhibition in two age groups, two hours after estradiol benzoate injection in bilaterally ovariectomized and adrenalectomized rats. 20 day-old, immature and 80 day-old adult rats were used (Fig. 16, 17) and estradiol benzoate at a dose of 1.0 µg/100 grams body weight was used as the feedback stimulus. 24 hours after the combined removal of the ovaries and the adrenals, circulating estradiol had declined to below assay sensitivity (< 15 pg/ml). The 20 day-old rats showed a significant increase in plasma estradiol 2 hours after steroid injection, as compared to their oil-treated controls. The 80 day-old rats did not show a significant increase in their circulating estradiol levels, 2 hours after steroid injection. The 80 day-old group (Fig. 17) also failed to show a significant plasma LH increase after surgery or a significant increase in plasma LH, 24 hours after ovariectomy and adrenalectomy. Circulating LH values were reduced more than 50% 2 hours after steroid injection, although this decline is not at the level of statistical significance. Although it was apparent that the younger rats were more estrogen-sensitive than the adult group, plasma estradiol 2 hours after steroid injection in immature rats was significantly higher than in the mature rats.

To examine the LH response to equal circulating estradiol levels, the above experiment was repeated with the 80 day-old rats receiving twice as much estradiol benzoate (1.0 µg/100 grams of body weight) as the 20

day-old rats (0.5 µg/100 grams of body weight). In this case, the plasma estradiol in each group was significantly higher than its respective oil-treated control, yet the levels were the same when comparison was made between the two groups (Figs. 18, 19). Although the estradiol levels obtained in the two identical experiments on 80 day-old rats (Fig. 17 and 19) are the same, the LH values seem to be different; these differences are not statistically significant and most probably represent variation in the LH radioimmunoassay. Two significant observations can be made in this experiment: ovariectomy and adrenalectomy in the young rats (Fig. 18) resulted in a 600% increase in plasma LH, whereas the older group showed only a 400% increase (Fig. 19); control LH levels were the same in both ages. The same circulating estradiol values caused a significant inhibition of LH secretion in the immature rats whereas it failed to significantly decrease plasma LH levels of the adult group. Assuming that the binding of estradiol to plasma proteins does not change (Ramaley, 1971), the above two observations are direct evidence of the difference in sensitivity of immature and adult rats to circulating estrogens.

As mentioned earlier, evidence exists to suggest either estradiol, in the adult rat (Shaikh et al., 1975), or some non-estradiol interferring substance, in the immature rat (Weisz and Gunsalus, 1973), as a contributing source for plasma "estradiol" measurements. In the present experiments, suggestive evidence exists to support the second possibility. If the two experiments in which 0.5 µg estradiol benzoate was injected into 20 day-old rats (Figs. 11 and 18) are compared, one sees almost identical plasma LH patterns; however, when the adrenals were left intact (Fig. 11) much higher "estradiol" levels were observed. Since the plasma LH responses are the same, the results suggest that whatever the adrenals contributed to the "estradiol" levels measured in the first case (Fig. 11), it was not biologically active as an estrogen.

Although the interference of an adrenal substance with estradiol measurements in Experiment 4 prevents a clear cut interpretation of the data, the fact still remains that the various age groups studied showed different sensitivities to a 0.5 µg/100 grams body weight injection of estradiol benzoate. More importantly, the LH response to castration has provided solid evidence for the higher sensitivity of the prepuberal rat compared with the adult animal.

Fig. 22 presents the combination of data from Fig. 21 a-d along with results from Fig. 20; the pattern of feedback sensitivity at each age group is equally evident when observation is made on either the response to steroid removal or to its replacement. The sensitivity to steroid removal is absent at age 5 days and increases to reach a peak at 15 days of age. The lack of an LH rise after ovariectomy in 5 day-old rats is in agreement with the report of Goldman et al (Goldman et al., 1971) who observed no rise in either LH or FSH after castration in neonatal female rats. There is a steady decline in the LH response to ovariectomy after day 15 which continues to day 80, the oldest age group studied. The feedback sensitivity to estrogen replacement is also absent at 5 days of age and does not become significantly developed until 15 days at which time the response is at its maximum. The rats show progressively lower sensitivity to the injected steroid after 15 days of age, until the response becomes absent at 40 to 80 days of age. These results confirm the report by Ramirez and McCann (Ramirez and McCann, 1963) who observed, using the ovarian ascorbic acid depletion assay, that immature animals were 2 to 3 times more sensitive to the feedback effect of estrogen than the adult.

One must correlate the circulating LH and "estradiol" levels obtained in intact rats in Exp. 4 with caution. There is a large contribution by the adrenals of an unknown substance to the estradiol levels of the young animals. We cannot be sure that once the adrenal contribution is eliminated

the pattern of ovarian estradiol will still remain the same, that is, with a peak at 15 days of age. It would seem that lack of feedback sensitivity, prior to 15 days of age, leads to a gradual increase in synthesis and release of gonadotropins. Once the animals start to become greatly responsive to estrogen feedback inhibition, that is, at 15 days of age, the gonadotropins start to decline and this relationship is maintained until the onset of puberty. However, this is a highly speculative explanation.

In conclusion, these experiments show that there is an increase in the synthesis and release of gonadotropins between 10 and 20 days of age; the reason for this observation is not clear at present. More importantly, they do indeed show that the young female rat is more sensitive to feedback inhibition of LH release by estrogen than is the adult rat. Whether this differential sensitivity to estrogen may account for the normal processes leading to the onset of puberty cannot be deduced from these experiments. The observation of a gradual decline in feedback sensitivity, starting at 15 days of age and continuing through puberty, seems to suggest that the onset of puberty is not due to a sharp decline in feedback sensitivity between estrogens and LH.

TABLE 1. EFFECT OF OVARIECTOMY ON PLASMA LH LEVELS IN ADULT RATS (80 days old)

GROUP	NO. OF RATS	PLASMA LH (ng/ml)
Intact adult rats	9	72 ± 3^{a}
5 hours after ovariectomy	9	78 ± 7^{b}
24 hours after ovariectomy	10	138 ± 30^{c}

a Mean $\pm$ SE

b P=0.42 vs intact

c P=0.055 vs intact

TABLE 2. RECOVERY OF KNOWN AMOUNTS OF ESTRADIOL-17B (E_2) ADDED TO 1.0 ML OF CHARCOAL-TREATED PLASMA

E_2 (pg) ADDED TO CHARCOAL-TREATED PLASMA	NO. OF DETERMINATIONS	MEAN (pg)	S.D.[a] (pg)	C.V.[b] (%)
0[c]	3	0.0	-	-
40[d]	5	43.8	2.77	6.3
75[d]	5	76.6	2.6	3.4
100[d]	5	101.0	0.81	0.8

a Standard deviation
b Coefficient of variation
c Volume of plasma = 1.0 ml
d Volume of standard = 0.1 ml

TABLE 3. 'ESTRADIOL' MEASURED IN DISTILLED WATER AND IN PLASMA FROM RATS UNDER VARIOUS EXPERIMENTAL CONDITIONS

TREATMENT	VOLUME (ml)	NO. OF DETERMINATIONS	MEAN (pg)	S.D.[a] (pg)
Distilled water	1.0	5	0	0
20 day-old female, intact	1.0	4	203	80
20 day-old female, 24 hours after ovariectomy + adrenalectomy	1.0	6	16	21
80 day-old female, intact	1.0	6	63	33
80 day-old female, 24 hours after ovariectomy + adrenalectomy	1.0	6	10	9
Charcoal-treated plasma	1.0	3	0	0

a Standard deviation

TABLE 4. ORGAN WEIGHTS IN FEMALE RATS BETWEEN 10 AND 20 DAYS OF AGE

AGE (days)	NO. OF RATS	ANTERIOR PITUITARY (mg)	OVARIES (mg)	UTERI (mg)	ADRENALS (mg)
10	15	1.54 ± 0.12[a]	7 ± 0.4[a]	8 ± 1.0[a]	5 ± 0.2[a]
11	15	1.70 ± 0.04	6 ± 0.3	9 ± 0.5	6 ± 0.2
12	15	1.80 ± 0.10	7 ± 0.2	10 ± 0.3	6 ± 0.3
13	15	1.77 ± 0.14	10 ± 0.5	16 ± 1.0	7 ± 0.5
14	15	2.15 ± 0.05	10 ± 0.3	14 ± 0.5	8 ± 0.4
15	9	2.28 ± 0.01	10 ± 0.3	14 ± 0.5	7 ± 0.1
16	15	2.76 ± 0.15	13 ± 0.1	17 ± 1.0	9 ± 0.4
17	15	2.57 ± 0.07	14 ± 0.5	17 ± 0.5	10 ± 0.5
18	15	2.37 ± 0.09	16 ± 1.0	17 ± 1.0	10 ± 0.4
19	15	2.55 ± 0.07	18 ± 1.0	17 ± 1.0	11 ± 0.3
20	15	2.94 ± 0.18	23 ± 1.0	17 ± 1.0	13 ± 0.4

a Mean ± S.E.

TABLE 5. PLASMA AND PITUITARY CONCENTRATIONS OF LH, FSH AND PROLACTIN IN FEMALE RATS BETWEEN 10 AND 20 DAYS OF AGE

AGE (days)	NO. OF RATS	PLASMA CONCENTRATION (ng/ml)[a]			ANT. PITUITARY CONCENTRATION (µg/mg)[a]		
		LH	FSH	PROLACTIN	LH	FSH	PROLACTIN
10	5	98 ± 12	1273 ± 130	8 ± 1	9 ± 1	7 ± 1	47 ± 6
11	5	91 ± 14	1080 ± 20	8 ± 1	12 ± 2	6 ± 1	56 ± 8
12	5	109 ± 16	1154 ± 93	7 ± 1	12 ± 2	7 ± 1	47 ± 10
13	5	134 ± 6	1645 ± 207	7 ± 1	18 ± 2	11 ± 1	61 ± 11
14	5	124 ± 32	1512 ± 131	10 ± 1	28 ± 3	13 ± 2	95 ± 5
15	5	99 ± 5	1124 ± 49	8 ± 2	12 ± 3	10 ± 2	77 ± 25
16	5	113 ± 6	1701 ± 138	9 ± 1	28 ± 2	14 ± 1	54 ± 5
17	5	129 ± 30	1237 ± 127	10 ± 1	23 ± 6	7 ± 2	61 ± 15
18	5	65 ± 4	608 ± 124	10 ± 1	23 ± 5	13 ± 1	137 ± 13
19	5	118 ± 17	678 ± 43	13 ± 2	16 ± 6	17 ± 1	129 ± 7
20	5	68 ± 12	349 ± 24	8 ± 1	20 ± 3	17 ± 2	151 ± 7

a Mean ± S.E.

TABLE 6. CIRCULATING ESTRADIOL LEVELS IN RESPONSE TO ESTRADIOL BENZOATE ADMINISTERED (SUBCUTANEOUSLY) IN OVARIECTOMIZED ADULT RATS (n = 3)

DOSE OF ESTRADIOL BENZOATE (per 100 grams, body weight)	PLASMA ESTRADIOL (pg/ml)[a]				
	24 HR AFTER OVARIECTOMY	HOURS AFTER ESTRADIOL BENZOATE			
		1	2	3	4
5 µg	5 ± 5	1207 ± 452	658 ± 396	255 ± 206	108 ± 18
0.8 µg	14 ± 1	87 ± 13	85 ± 8	34 ± 1	26 ± 4
0.5 µg	35 ± 5	30 ± 4	33 ± 4	47 ± 11	-----
0.1 µg	27 ± 15	16 ± 3	24 ± 7	21 ± 8	23 ± 12

a Mean ± S.E.

TABLE 7. CROSS-REACTION OF SELECTED STEROIDS WITH THE ESTRADIOL ANTISERUM (from Nett, et al., 1973)

COMPOUND	PERCENT CROSS-REACTION[a]
Estradiol-17B	100
Estrone	62
Estradiol-17a	33
Estriol	27
Equilin	17
Equilenin	19
Testosterone	0
Androstenedione	0
Progesterone	0
20B-Hydroxypregn-4-en-3-one	0
Corticosterone	0
Cortisol	0

a The percent cross-reaction was defined as the pg of estradiol-17B equivalent exhibited by 100 pg of the steroid listed.

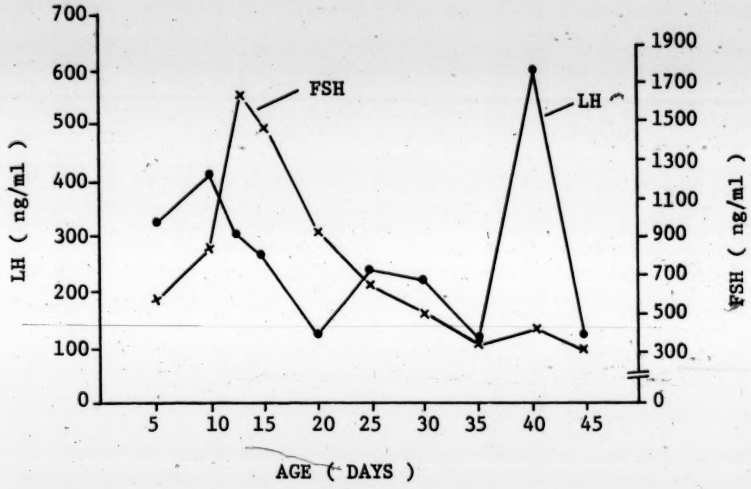

Fig.1A Data from Ojeda and Ramirez, 1972.

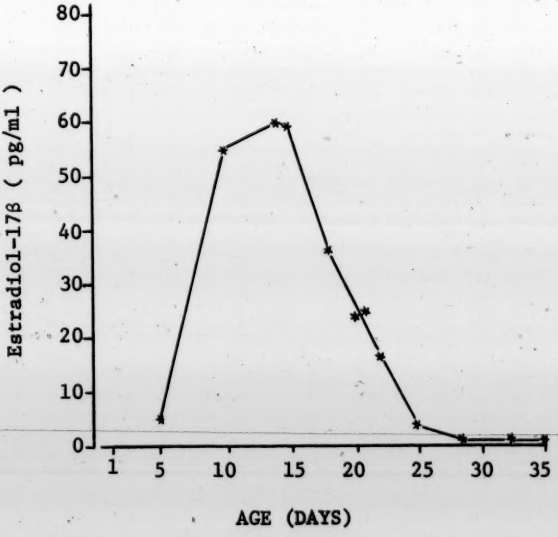

Fig.1B Data from Meijs-Roelofs et al., 1973.

Fig.1 Plasma LH, FSH and estradiol-17β in growing immature female rats.

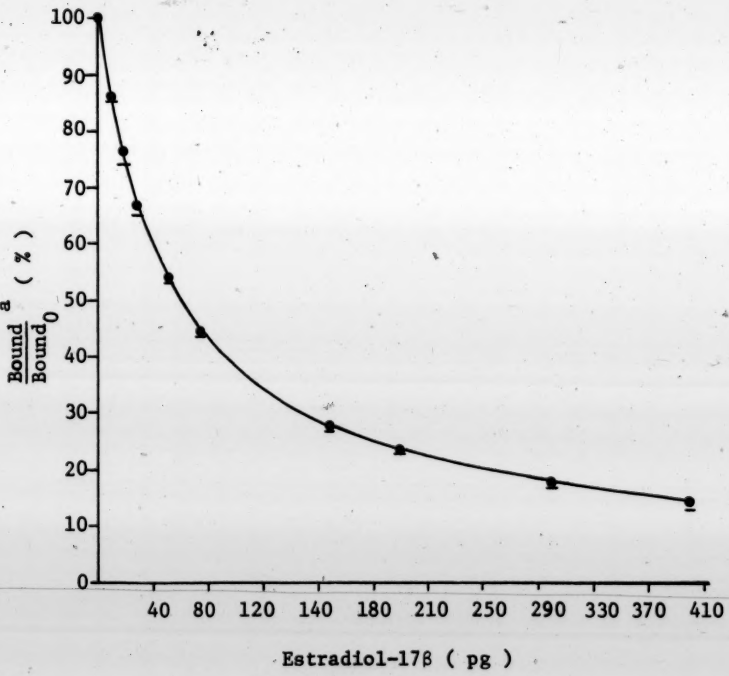

Fig.2 Standard curve for the estrogen radioimmunoassay (n=3).

a Binding of unlabelled estradiol, as percent of binding in the absence of any unlabelled steroid.

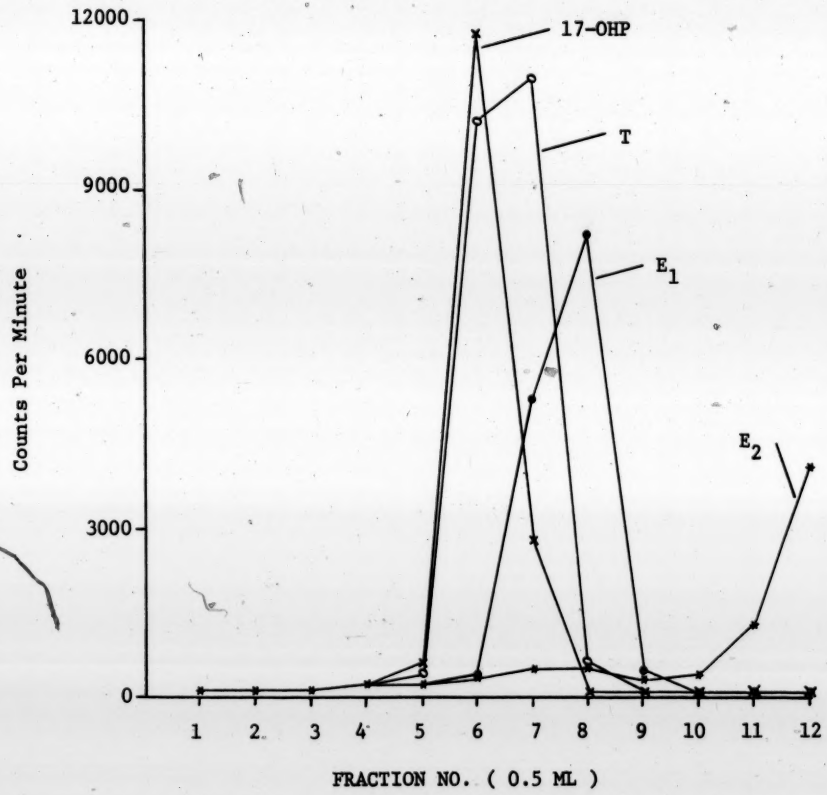

Fig.3 Elution patterns of radioactively-labelled estradiol-17β (E_2), estrone (E_1), testosterone (T) and 17-Hydroxyprogesterone (17-OHP) through a 15 Cm column of Sephadex LH-20.

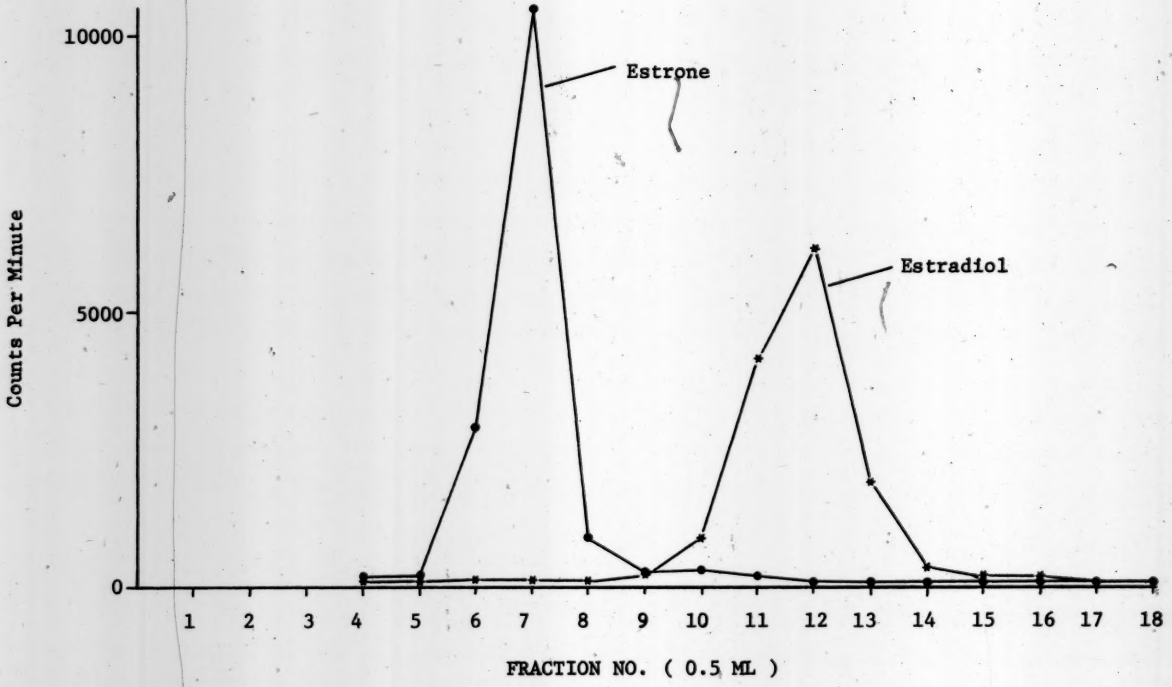

Fig.4 Elution patterns of radioactively-labelled estradiol and estrone through a 9 Cm column of Sephadex LH-20.

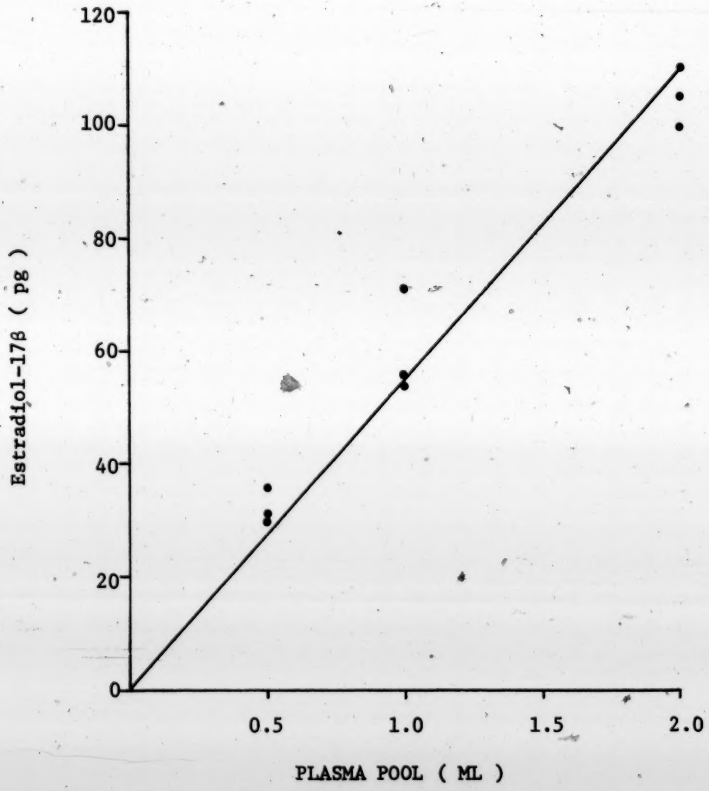

Fig.5 Estradiol-17β measured in a serially diluted plasma pool.

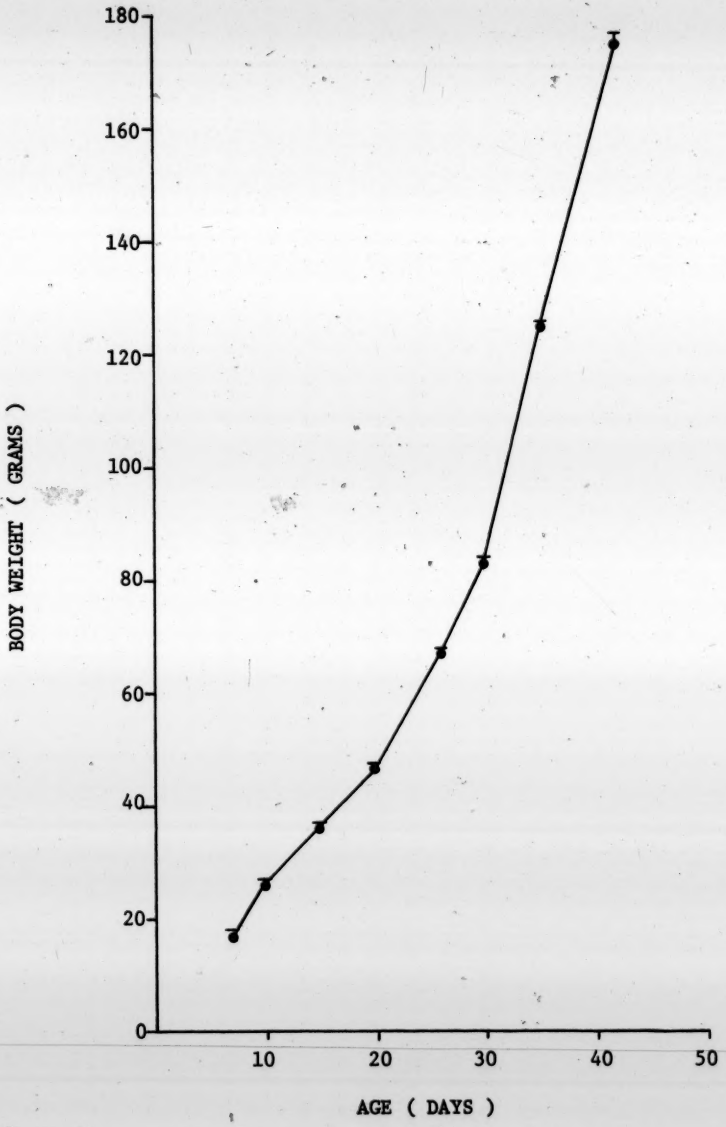

Fig.6 Body weight as a function of age in female rats (n=15).

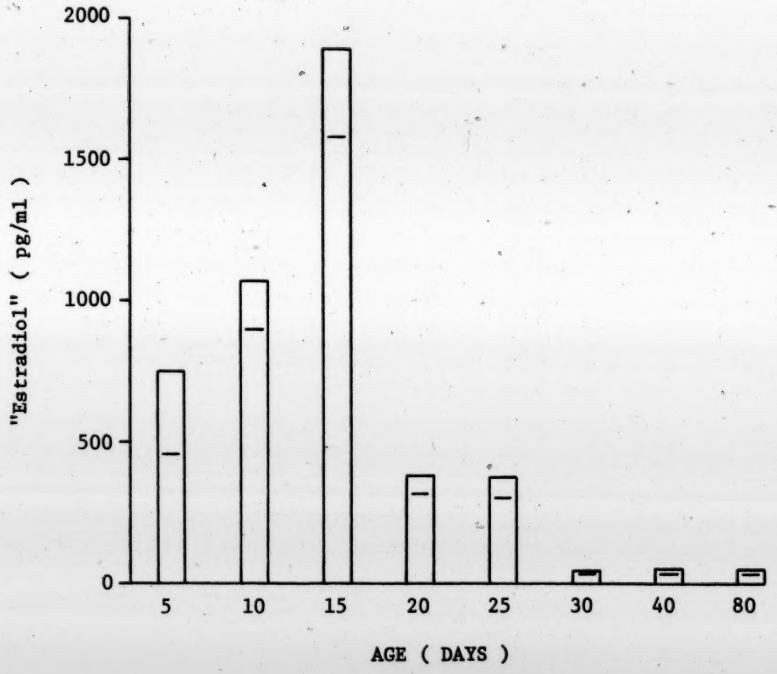

Fig.7 Plasma "estradiol" levels during development in female rats.

AGE: 5 DAYS

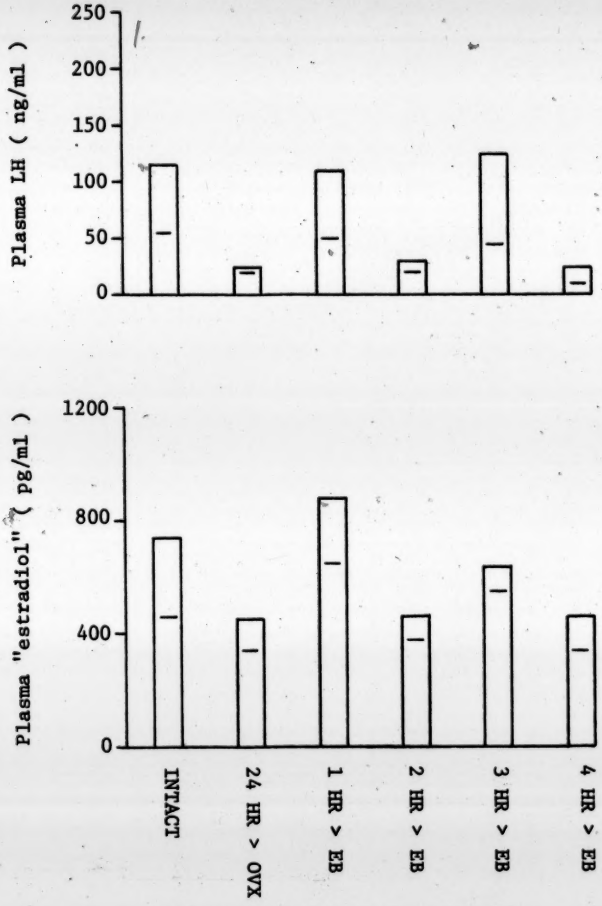

Fig.8 Plasma levels of LH and "estradiol" in intact, ovariectomized (OVX) and estradiol benzoate[a] (EB)-treated ovariectomized rats[b] (n=5).

a 0.5 µg/100 grams body weight
b 5 day-old rats

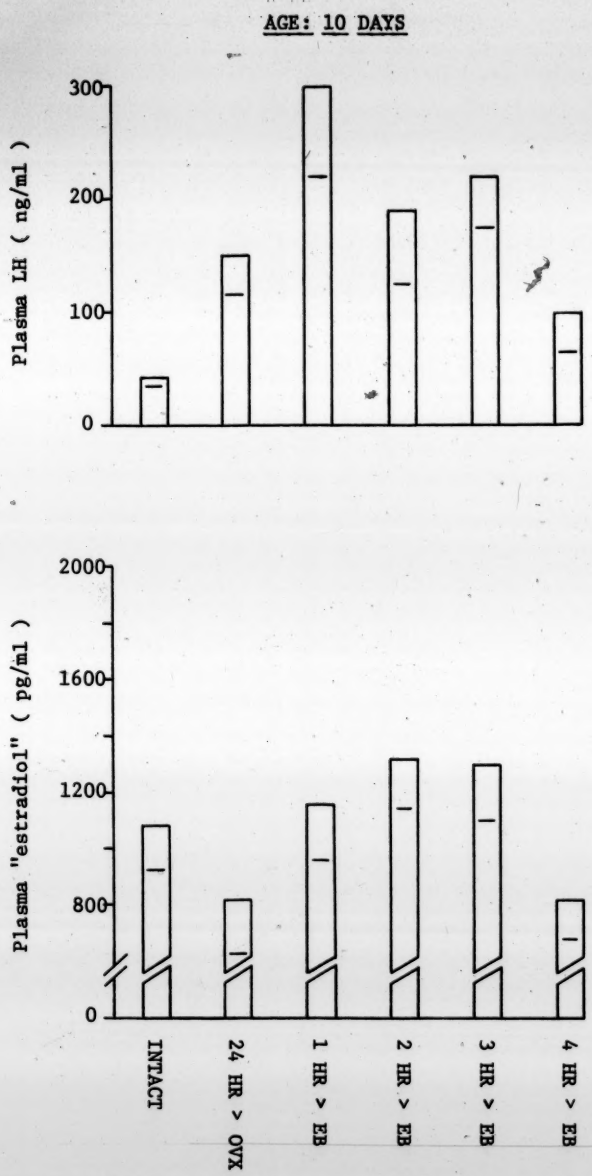

Fig.9 Plasma levels of LH and "estradiol" in intact, ovariectomized (OVX) and estradiol benzoate[a] (EB)-treated ovariectomized rats[b] (n=7).

a 0.5 µg/100 grams body weight
b 10 day-old rats

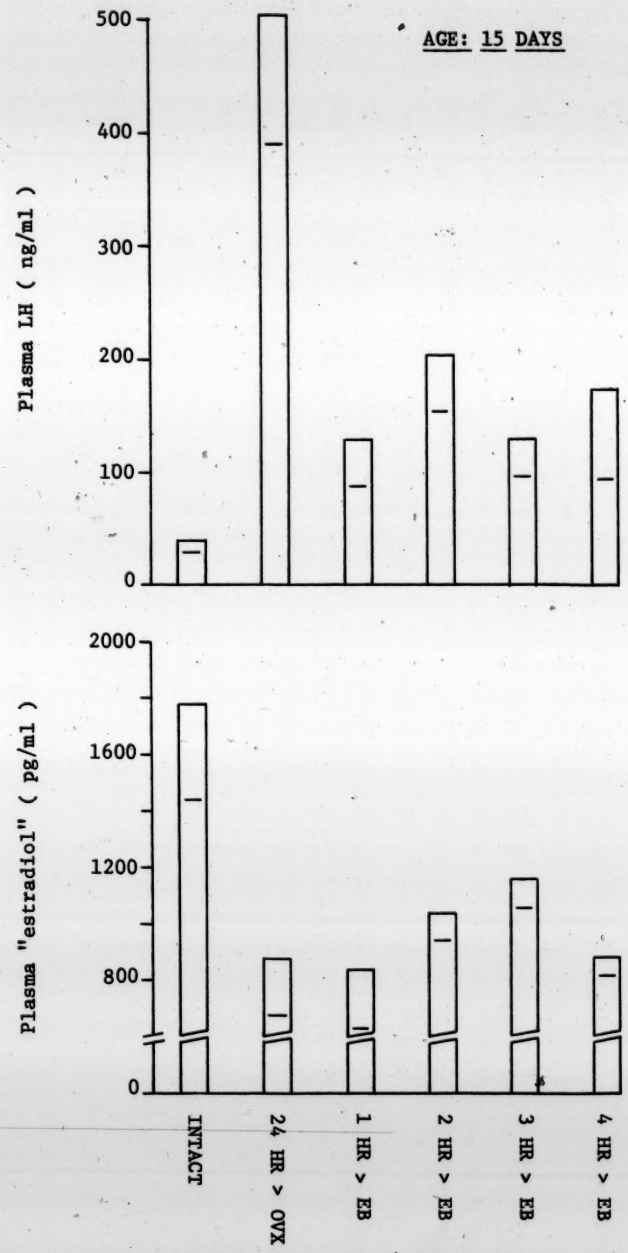

Fig.10 Plasma levels of LH and "estradiol" in intact, ovariectomized (OVX) and estradiol benzoate[a] (EB)-treated ovariectomized rats[b] (n=5).

a 0.5 µg/100 grams body weight
b 15 day-old rats

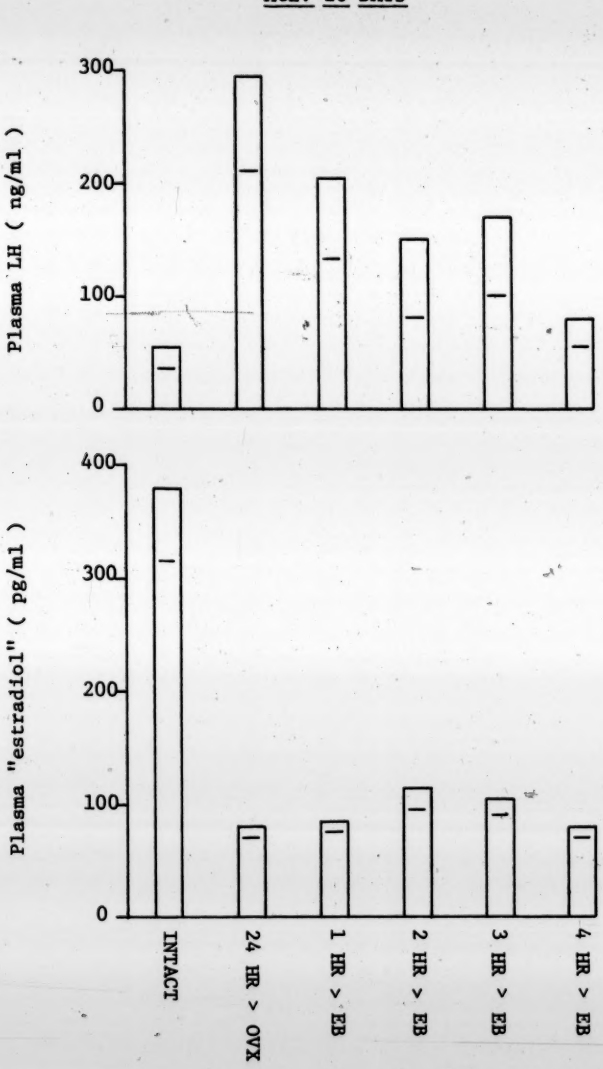

Fig.11 Plasma levels of LH and "estradiol" in intact, ovariectomized (OVX) and estradiol benzoate[a] (EB)-treated ovariectomized rats[b] (n=6).

a 0.5 µg/100 grams body weight
b 20 day-old rats

AGE: 25 DAYS

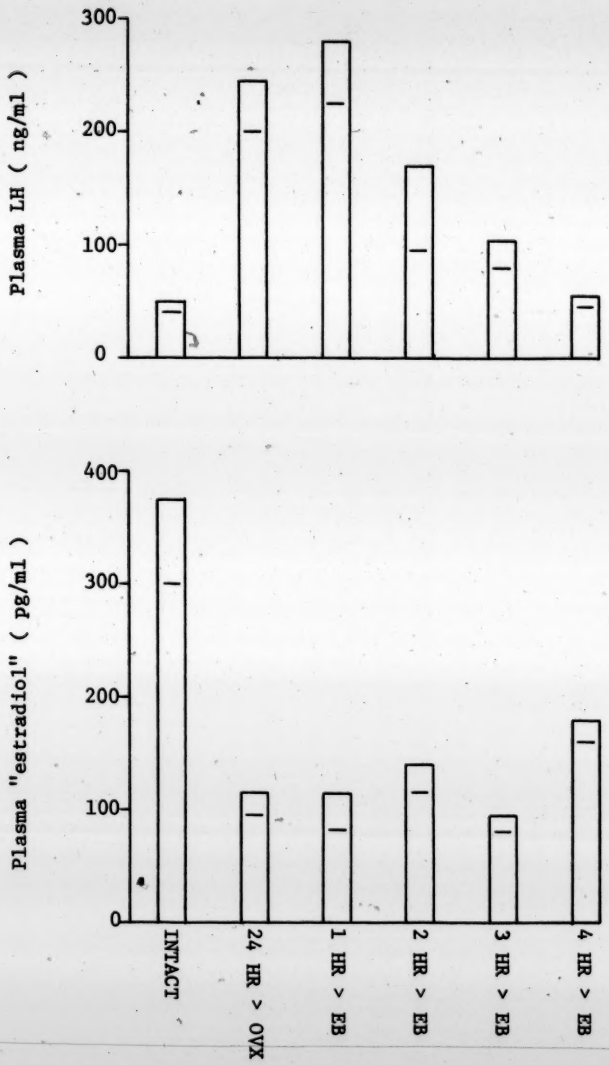

Fig.12 Plasma levels of LH and "estradiol" in intact, ovariectomized (OVX) and estradiol benzoate[a] (EB)-treated ovariectomized rats[b] (n=6).

a 0.5 µg/100 grams body weight
b 25 day-old rats

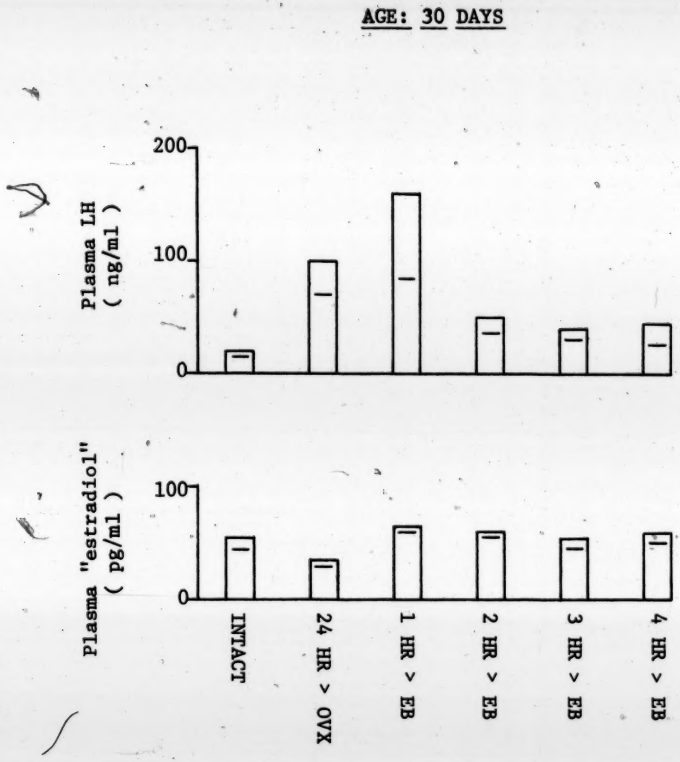

Fig. 13 Plasma levels of LH and "estradiol" in intact, ovariectomozed (OVX) and estradiol benzoate[a] (EB)-treated ovariectomized rats[b] (n=6).

a 0.5 µg/100 grams body weight
b 30 day-old rats

AGE: 40 DAYS

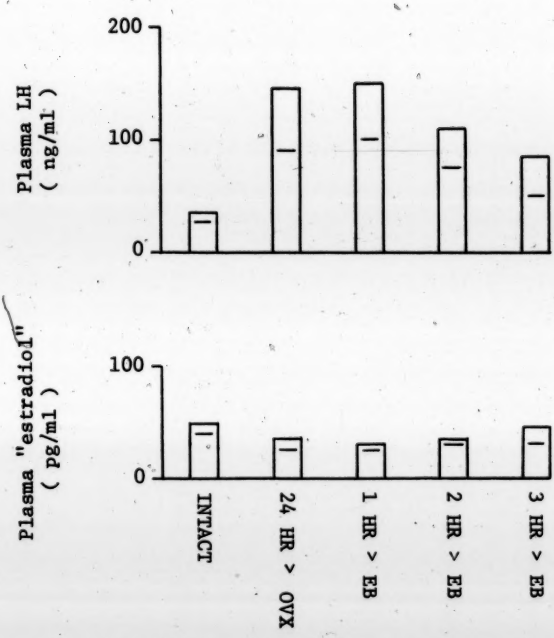

Fig.14 Plasma levels of LH and "estradiol" in intact, ovariectomized (OVX) and estradiol benzoate[a] (EB)-treated ovariectomized rats[b] (n=8).

a 0.5 µg/100 grams body weight
b 40 day-old rats

AGE: 80 DAYS

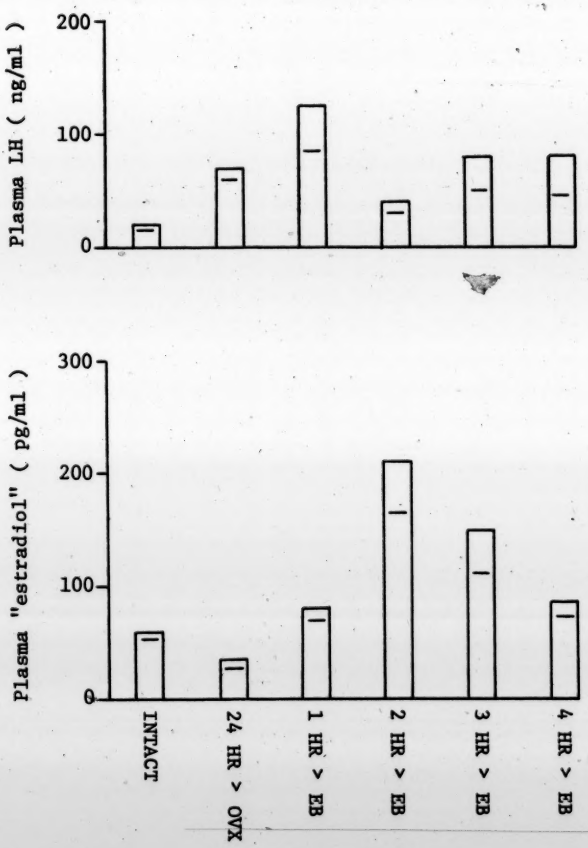

Fig.15 Plasma levels of LH and "estradiol" in intact, ovariectomized (OVX) and estradiol benzoate[a] (EB)-treated ovariectomized rats[b] (n=5).

a 0.5 µg/100 grams body weight
b 80 day-old rats

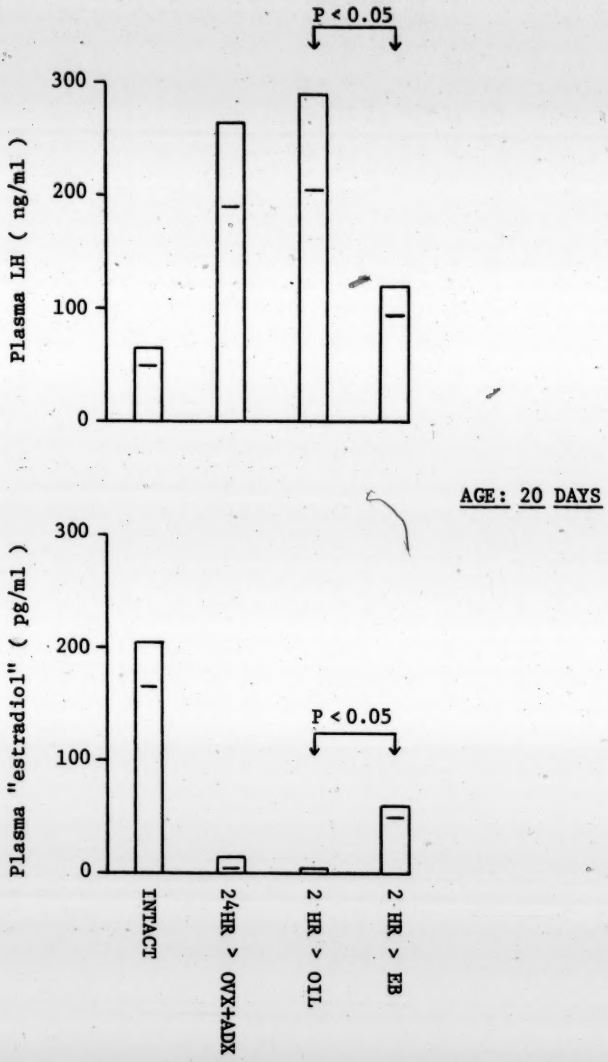

Fig.16 Plasma levels of LH and "estradiol" in intact, ovariectomized+adrenalectomized (OVX+ADX) and estradiol benzoate[a] (EB)- or oil-treated OVX+ADX rats[b] (n=8).

a 1.0 µg/100 grams body weight
b 20 day-old rats

AGE: 80 DAYS

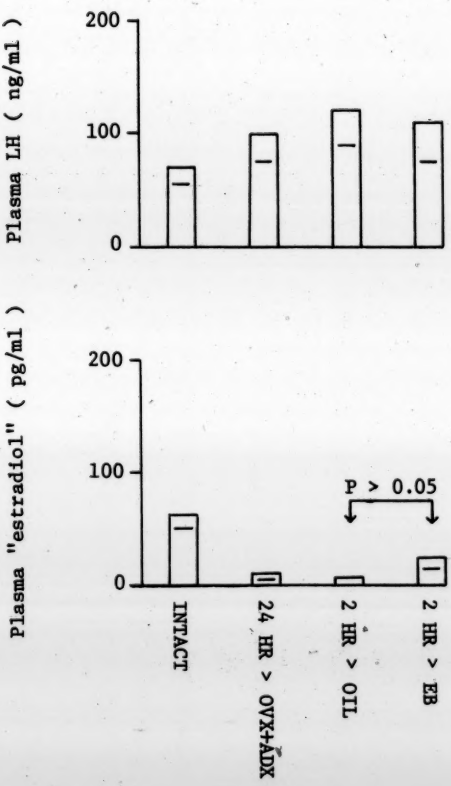

Fig.17 Plasma levels of LH and "estradiol" in intact, ovariectomized+adrenalectomized (OVX+ADX) and estradiol benzoate[a] (EB)- or oil-treated OVX+ADX rats[b] (n=8).

a 1.0 µg/100 grams body weight
b 80 day-old rats

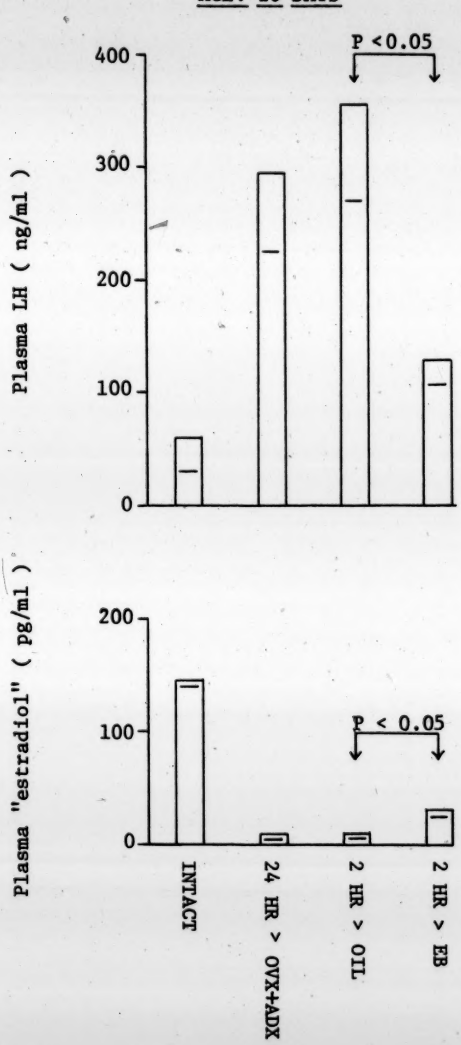

Fig.18 Plasma levels of LH and "estradiol" in intact, ovariectomized+adrenalectomized (OVX+ADX) and estradiol benzoate[a] (EB)- or oil-treated OVX+ADX rats[b] (n=6).

a 0.5 µg/100 grams body weight
b 20 day-old rats

AGE: 80 DAYS

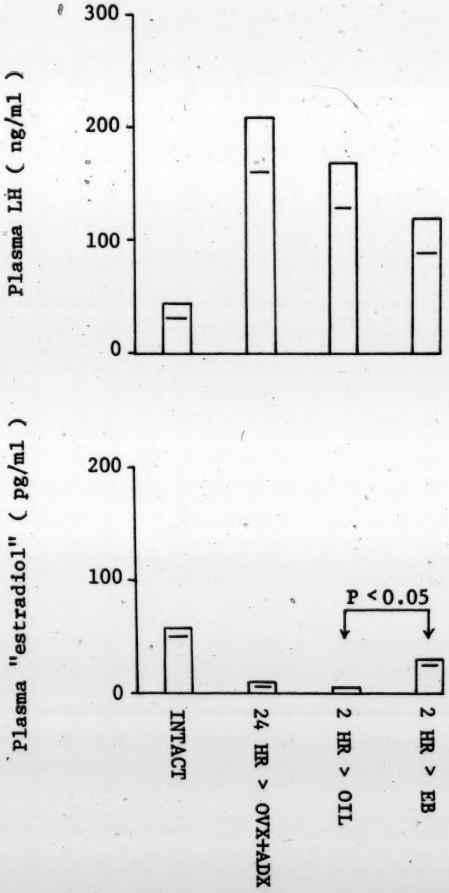

Fig.19 Plasma levels of LH and "estradiol" in intact, ovariectomized+adrenalectomized (OVX+ADX) and estradiol benzoate[a] (EB)- or oil-treated OVX+ADX rats[b] (n=6).

a 1.0 µg/100 grams body weight
b 80 day-old rats

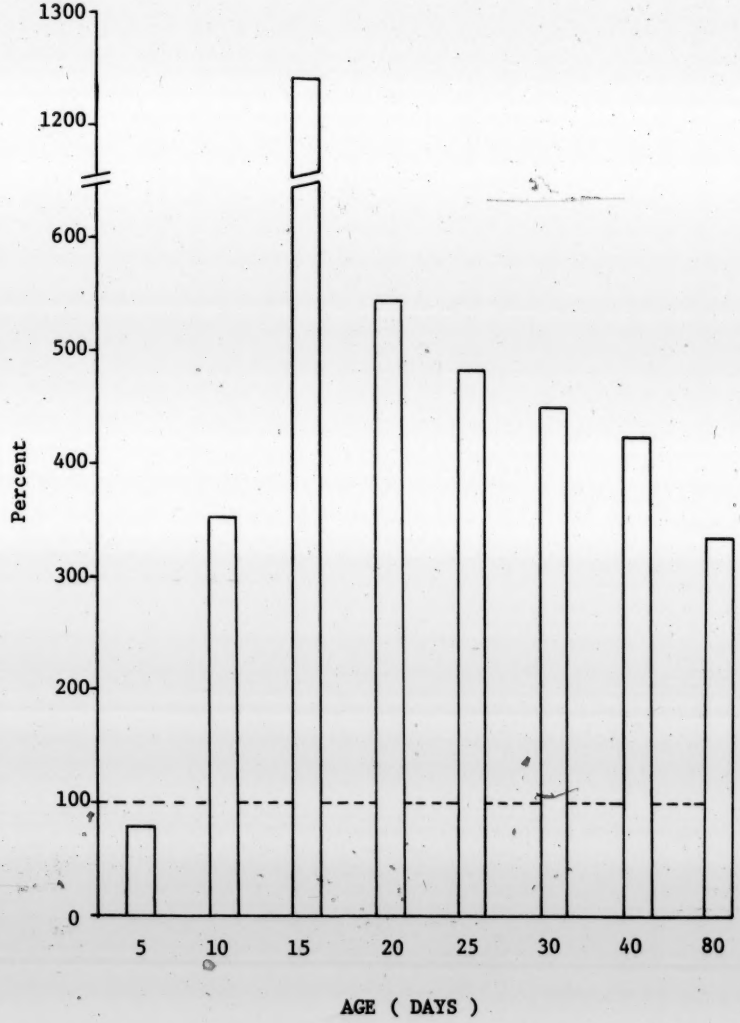

Fig.20 Plasma LH 24 hours after ovariectomy as percent of respective intact controls.

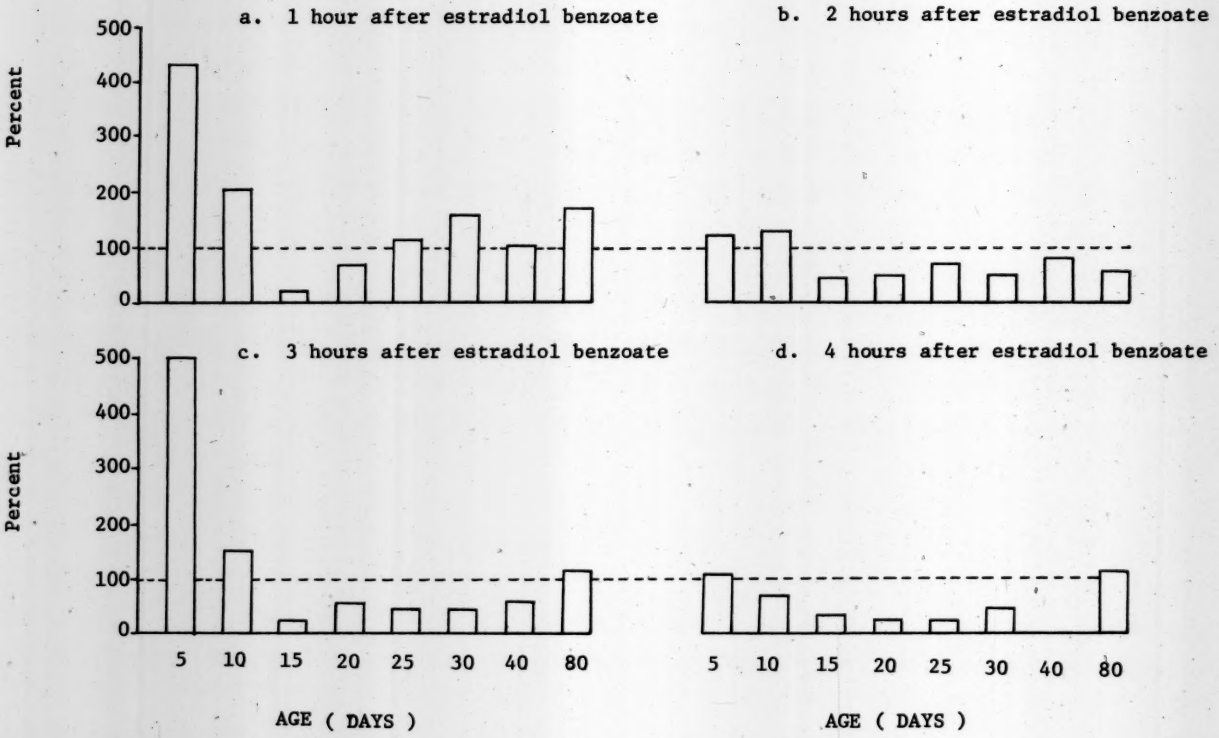

Fig.21 Plasma LH levels at various times after steroid administration, as percent of the respective pre-injection controls.

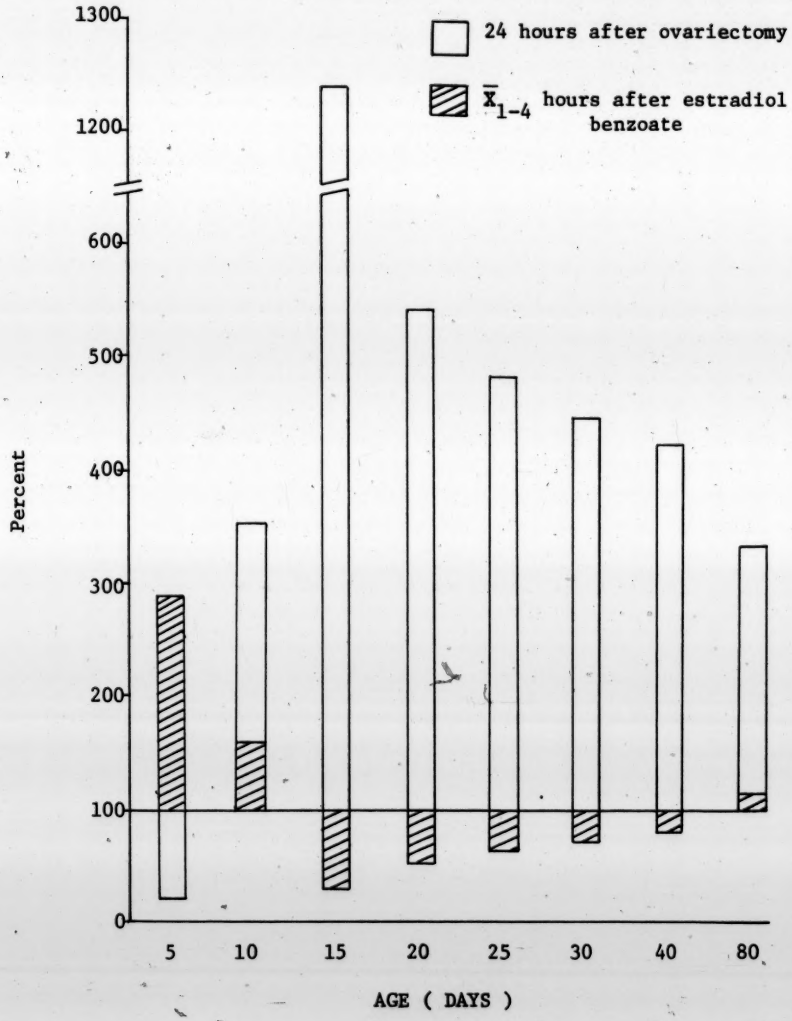

Fig.22 Plasma LH in response to ovariectomy and to estradiol benzoate administration, as percent of the respective pretreatment controls.

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